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NATURAL ANTIFUNGAL SYSTEMS FOR PREVENTION OF MOULD SPOILAGE IN BAKERY PRODUCTS

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ABSTRACT

Growth of spoilage fungi in bread and other bakery products is currently controlled with the addition of weak acid preservatives. Consumers demand more natural products and thus there is a need to reduce the amount of chemical preservatives added to foods, or to identify alternative, more 'natural' antifungal compounds with strong biological activity. This thesis reports on several areas of research undertaken in the project: evaluation of sub-optimal concentrations of existing preservatives, under different environmental conditions, on growth and ochratoxin A (OTA) production by six bread spoilage moulds; efficacy of new/natural antifungal compounds for possible use in bread preservation; evaluation of the impact of preservation hurdles on ecophysiology of the spoilage fungi, including niche overlap; and mechanisms of action of preservatives on hydrolytic enzymes.

It was found that the use of currently applied levels of the existing preservatives potassium sorbate, calcium propionate and sodium benzoate were effective, under low pH environments (pH 4.5) at completely controlling growth of spoilage moulds (*Aspergillus ochraceus, Eurotium repens, Cladosporium herbarum, Penicillium corylophilum* and *Penicillium verrucosum*) on wheat flour-based substrates over a period of 30 days. At higher pH levels, the efficacy decreased being almost nil at pH 7.5. The use of sub-optimal concentrations of weak acid preservatives led in most cases to reductions in lag times and/or stimulation of mould growth and ochratoxin A production by *P.verrucosum* strains.

Four different antioxidants and twenty plant essential oils were screened for in vitro antifungal activity. Antioxidants butylated hydroxyanisole (BHA) and *p*-hydroxybenzoate (propyl paraben) and essential oils of clove, cinnamon, bay and thyme showed the strongest antifungal activity, and were therefore considered as possible alternative compounds for bread preservation. Under different environmental conditions MIC (Minimum Inhibitory Concentration) values ranged from 200-500 ppm (w/w) for antioxidants and from 200-1000 ppm for essential oils. In contrast to weak organic acids, the antifungal efficacy of these alternative preservatives was less dependent on the pH of the media.

The addition of antioxidants and essential oils to bread analogues resulted in a significant decrease in their biological activity, probably due to preservative molecules binding to food components such as lipids, proteins or polysaccharides. Maximum doses studied (1000 ppm) had no effect on mould growth of the species tested.

On wheat flour agar *A.ochraceus* and three strains of *P.verrucosum* did not produce any OTA at 25°C on a range of a_w x temperature conditions and 54 days incubation. However, on bread analogues, *P.verrucosum* strains produced up to 75 ppm after 28 days. *A.ochraceus* produced no OTA on bread analogues. Stimulation of OTA production was observed on both substrates by the use of sub-optimal concentrations of existing preservatives. Conversely, although growth of *P.verrucosum* on bread analogues was not inhibited by the addition of 1000 ppm of antioxidants and essential oils, a significant reduction in inhibition on OTA was found.

Nutritional assimilation by spoilage moulds of 32 key bread carbon sources, under different environmental factors and in the presence of existing preservatives, was determined for the first time. Niche sizes and niche overlap indices (NOI) varied with species, environmental factors (a_w, pH and temperature) and with the presence of preservatives. Overall, the addition of potassium sorbate increased the inter-specific competition for nutrients.

Lastly, the impact of environmental conditions and existing/alternative preservatives on hydrolytic enzyme production by moulds colonising wheat flour-based substrates was studied. Of seven different enzymes assayed, only β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase were detected. The type and level of enzyme produced varied with species and was significantly affected by time of incubation, a_w and by the presence of preservatives.

From the results presented in this thesis it can be concluded that antioxidants and essential oils are promising alternative compounds for controlling growth and toxin production of common bread spoilage moulds. The effectiveness of these compounds will be dependent on other factors such as the mode in which they are applied and the

A la mareta,

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ABREVIATION LIST

pres	Preservative
eso	Essential oil
ant	Antioxidant
WFA	Wheat Flour Agar
MEA	Malt Extract Agar
e.r.h.	Equilibrium Relative Humidity
a_w	Water Activity
BHT	Butylated Hydroxytoluene
BHA	Butylated Hydroxyanisole
PP	Propyl *p*-Hydroxybenzoate (Propyl Paraben)
OTA	Ochratoxin A
OTB	Ochratoxin B
HPLC	High Performance Liquid Chromatography
CS	Carbon Sources
NOI	Niche Overlap Index
Spp	Species
CP	Calcium Propionate
SB	Sodium Benzoate
PS	Potassium Sorbate
AO	*Aspergillus ochraceus*
CH	*Cladosporium herbarum*
ERE	*Eurotium repens*
PC	*Penicillium corylophilum*
PR	*Penicillium roquefortii*
M450	*Penicillium verrucosum* strain M450
M453	*Penicillium verrucosum* strain M453
PV3	*Penicillium verrucosum* strain PV3

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Chapter 1

LITERATURE REVIEW

1.1 FOOD SPOILAGE

The quality of foods can be adversely affected by physical, chemical, biochemical and microbiological processes. Spoilage of foods by undesirable microbial contamination is of major importance in the food industry both economically and in terms of public health. Microbial food spoilage costs the food industry many millions of Euros annually (Roller, 1999), and some of the common food spoilage microorganisms can cause diseases and produce secondary metabolites that are toxic to animals and man.

Food-borne diseases and microbial spoilage of food results from the failure or inability to control microorganisms at one or more stages of the food chain, from raw material production to consumption of the final product. Fungi are one of the major groups of microorganisms that colonise and deteriorate foodstuffs. Almost all food commodities can be spoiled by fungi because of their ubiquitous nature and ability for growth over a wide range of environmental conditions.

1.1.1 Parameters affecting fungal growth

The type and level of fungal contamination in a food matrix is affected by several factors (1) intrinsic factors depending on the characteristics of the substrate, the food itself (e.g., pH, nutrients or the amount of available water), (2) processing factors resulting from the processes applied during the manufacturing of the food (e.g. heating, irradiation, chemical addition), (3) extrinsic factors, imposed from the outside (e.g., temperature and humidity of storage atmosphere) or (4) implicit factors that depend on the particular dominant microbial flora that develops in response to other factors (Mossel *et al.*, 1995). These factors are all hurdles which the spoilage microorganisms need to overcome for effective growth and survival.

(a) **Water availability**

Water availability is one of the most important factors determining mould spoilage. All moulds require a source of water for their correct functioning since ultimately, reactions require an aqueous environment. This parameter is an important intrinsic, processing or extrinsic factor. The water availability in the food (intrinsic factor) will determine the type and level of fungal colonisation. Reduction of the water availability of the substrate e.g. by adding salts (processing factor) and/or controlling the humidity of the storage environment (extrinsic factor) are useful measures commonly taken to control mould contamination and growth on the foodstuff (Mossel *et al.*, 1995).

Water is an important constituent of all food and the availability of this water in the food is important in determining its susceptibility to fungal attack. In a substrate not all the water content is readily available for fungal growth. A proportion of the water content is strongly bound to the substrate (the water of constitution), and the more weakly bound water (free water) is more readily available for fungal growth (Magan, 1997).

There are two main parameters commonly used as an expression of the water availability in a substrate, *water potential* (Ψ) mainly used in soil microbiology and *water activity* (a_w) generally used in foodstuffs (Magan, 1997). Water activity is defined as the ratio of the vapour pressure of the substrate at equilibrium with the atmosphere, to that of pure water at the same temperature and pressure. Thus the water activity of pure water is 1 and 0 for a completely dehydrated food. Although a_w is the parameter generally accepted and used in all food commodities, certain authors have questioned its validity in low-moisture and intermediate-moisture foods since these kind of foods may be very sensitive to changes in moisture so the measured vapour pressure of water is no longer in equilibrium (Rahman and Labuza, 1999).

The a_w level of the food is of practical importance as it controls the onset and severity of mould spoilage. Foods more likely to show rapid deterioration due to biological and chemical changes are usually those with high a_w. For example, Abdullah *et al.* (2000)

reported a mould-free shelf-life of up to 6 months for flours of different origin when the a_w was maintained at 0.65 while at 0.98 a_w fungal contamination occurred within 15 days of storage.

In general, at a given temperature, the ability of microorganisms to grow is reduced as the a_w decreases. The minimum value of a_w for the growth of most common food spoilage moulds is 0.80 (Rahman and Labuza, 1999; Jay, 2000) although there are some specific moulds such as *Eurotium spp* that are able to grow at lower a_w levels (between 0.65-0.70). The range of a_w at which moulds can grow is affected by temperature and the nutritional status of the food (Jay, 2000). Abellana *et al.* (1999) studied the effect of a_w (0.75 to 0.90) and temperature (15-30°C) on the growth of different *Eurotium* species on sponge cake analogues. They reported that, at the highest temperature tested (30°C), the range of a_w conditions for growth was wider than at lower temperatures, being able to grow at the lowest a_w level tested, 0.75.

When a microorganism is placed in an environment with low a_w and thus high osmotic pressure, the cell loses water by osmosis therefore affecting nutrient uptake, protein biosynthesis and enzyme activity (Mossel *at al.*, 1995; Magan, 1997; Rahman and Labuza, 1999). There are, however, some cell mechanisms that allow microorganisms to survive in a low a_w environment. One of the most important mechanisms is the synthesis and accumulation of compatible intracellular solutes that balance the osmolality and enable enzymes to function (Mossel *et al.*, 1995; Magan, 1997; Roller, 1999). In moulds, the key compatible solutes are polyhydric alcohols such as mannitol, glycerol, sorbitol and erythritol (Mossel *et al.*,1995; Rahman and Labuza, 1999).

Fungal responses to dry environments can be different depending on the solutes used to lower the a_w (Mossel, 1975; Christian, 1981; Rahman, 1999) and may affect the lowest limit of a_w at which the mould can grow. Hefnawy *et al.* (1999) found a lower growth rate of *Penicillium corylophilum* in liquid medium when the a_w of the substrate was lowered with NaCl compared to when glycerol was used.

The general effect of lowering a_w below the optimum level is to increase the length of the lag phase before fungal growth, and to decrease the growth rate and extent of colonisation, thus reducing the amount and impact of fungal contamination in the foodstuff. It is, therefore, essential to control humidity levels in food storage. This is particularly relevant to foods packaged in semi-permeable membranes as they will collect or lose moisture depending on the internal relative humidity with respect to the external.

A_w also has an influence over the effect of other environmental growth parameters such as pH, temperature and the oxidation-reduction potential (Eh). For instance, if the a_w is reduced below the optimum level, the pH values over which many fungi can grow becomes narrower and the minimum temperature for growth increases (Mossel *et al.*, 1995).

(b) Other factors

Although water availability is one of the most important factors that affects fungal growth on foodstuffs, other intrinsic factors such as pH, or the nutritional status of the food should also be considered in order to determine the possible impact and type of contamination. Temperature, pH and a_w are interacting factors important in hurdle technology approaches (see section 1.2.5) to control mould growth in food substrates.

pH is an important factor for food stability and preservation since hydrogen ions regulate many chemical, biochemical and microbiological reactions (Rahman, 1999). The internal pH of the microbial cell tends to be stable. Very low pH values adversely affect the cell integrity by inactivating one or more essential enzymes and reducing the transport systems for essential ions and nutrients (Rahman, 1999). The optimal range of pH at which a species can grow is often fairly narrow, so a small change in pH may markedly reduce the growth rate. This is an important point when preserving food. The ability of food to resist changes in pH is also a key factor in determining its spoilage.

The maximum and minimum value of pH for growth is again dependent on other factors such as a_w, temperature or the type of acid used. Citric, phosphoric and tartaric acids for example, lead to growth at a lower pH than when acetic or lactic acids are present (Jay, 2000).

The properties of the environment where the food is stored (extrinsic factors) affect both the food and the relevant microorganisms. Storage temperature and relative humidity, type and concentration of gases and presence and activities of other microorganisms are the extrinsic factors of greatest importance in food spoilage (Rahman, 1999). Control of the extrinsic factors is essential in food preservation taking into account not only the microbiological status of the food but also its physical and chemical quality.

1.1.2 Mycotoxin production in foodstuffs

One of the most important aspects of mould spoilage of foods is the formation of secondary metabolites that are toxic for animals and man, i.e. mycotoxins. Toxin production in foodstuffs is a worldwide problem mainly because moulds are ubiquitous and will readily colonise most foodstuffs if conditions allow. The problem is greater in some parts of the world because their climatic conditions are more favourable for growth of toxigenic fungi and thus, for synthesis of mycotoxins, but this is also much related to the care taken in producing, processing and storing the foodstuff (Marth, 1990). In fact, the incidence of mycotoxins in human health may be more pronounced in tropical developing countries than in continental Europe (Moss, 1994).

Mycotoxins are secondary metabolites of low molecular weight, produced by filamentous fungi usually in the late exponential or early stationary phase of their growth. Without growth, mycotoxins are not likely to be produced (Bullerman *et al.*, 1984). Mycotoxins are relatively stable molecules and thus can pass from the spoiled raw material through the food chain and be present in food products that have not been colonised by mould growth (Moss, 1989)

As in fungal growth, environmental conditions and the nutritional status of the food play an important role in toxin formation although toxigenesis, at least for most of the known mycotoxins, is much more dependent on the chemical composition of the substrate than is growth.

Generally, mycotoxin production is favoured by conditions of high humidity and a_w (Bullerman *et al.*, 1984) and by the presence of carbohydrates and/or lipids in significant quantities. The optimum a_w value for toxin production by toxigenic fungi ranges between 0.93-0.98 (Marth, 1990; Moss, 1996). The ability of moulds to produce mycotoxins under conditions of a_w stress also depends upon the strain, nutrient availability, temperature, oxygen tension and pH (Beuchat, 1983; Bullerman *et al.*, 1984).

There are more than 400 toxins produced by moulds, with aflatoxin probably being the best known (Filtenborg *et al.*, 1996). Although the ability to produce mycotoxins is widespread among food spoilage moulds, *Aspergillus*, *Penicillium* and *Fusarium* are three of the most important mycotoxigenic genera found in foodstuffs (Moss, 1989; Marth, 1990). Some of the most significant mycotoxins in the world are produced by these genera: fumonisins by *Fusarium* section *liseola* spp., aflatoxins by *Aspergillus flavus* and closely related species, and ochratoxin A produced almost exclusively by *Aspergillus ochraceus* and *Penicillium verrucosum* (Pitt, 1994).

Ochratoxin A

Ochratoxin A (OTA; $C_{20}H_{18}ClNO_3$) is a toxic metabolite (mycotoxin) produced by *Penicillium verrucosum* and some species of *Aspergillus*, particularly *Aspergillus ochraceus* (Skrinjar and Dimic, 1992; Simon, 1996; Moss, 1996; Varga *et al.*, 1996). Ochratoxin A was originally described from *Aspergillus ochraceus* and closely related species (Pitt, 1994) associated with mouldy legumes and cereal products (Marth, 1990). In European countries with temperate climates, the major source of OTA appears to be the species *Penicillium verrucosum* (Pitt, 1994).

Some authors have also reported other fungal species such as *Aspergillus niger* (Abarca *et al.*, 1994; Taniwaki *et al.*, 2003), *A.carbonarius* (Taniwaki *et al*, 2003) or *Eurotium* spp. (Kriŋvobok *et al.*,1995) as OTA producers in foodstuffs.

OTA is unstable in light and air, degrading when exposed to light especially under humid conditions. Ethanol solutions are stable for longer than 1 year if kept in adequate conditions. On the other hand, OTA is stable to heat (Boudra *et al.*, 1995) and will probably survive most physical food processing to some extent. Thus OTA may occur in food products made or derived from contaminated grain.

OTA occurrence in food, feed and related products have been extensively reported (see Delas *et al.*, 1995). However, cereal products are the major group of food commodities where the toxin is of greatest impact. OTA occurrence on bread, green coffee beans and other plant products such as barley or wheat during storage is a serious health hazard throughout the world (Pitt, 1994; Varga *et al.*, 1996; Taniwaki *et al.*, 2003).

As for other toxins, many factors such as a_w, temperature, fungal strain or the type of substrate affect OTA production in a certain foodstuff. Ramos *et al.* (1998), for example, studied the effect of different levels of a_w and temperature on the OTA production by three different strains of *Aspergillus ochraceus* on barley extract media and barley grains. They found a maximum OTA content when the producing fungi were grown over the a_w range, 0.96-0.98, and at 25-30°C. However, Delas *et al.* (1995), in a similar study on corn grain, established the optimum temperature for OTA production at 20°C and found a reduction in the OTA content when the temperature was increased from 20 to 25°C.

1.2 FOOD PRESERVATION

Food preservation, in the broader sense of the term, refers to all measures taken against any spoilage of food. In its narrower sense, however, food preservation denotes the measures taken against food spoilage due to microbial action (Lück and Jager, 1997).

1.2.1 Preservation techniques

Microbiological spoilage of foods can be controlled by different preservation procedures. The wide range of existing and new techniques used in food preservation act on three different fronts (Mossel *et al.*; 1995, Gould, 1996a; Rahman, 1999):

(i) restricting the access of microorganisms to the product
(ii) inactivating microorganisms
(iii) inhibiting growth of food spoilage microorganisms.

Access of fungi to certain foodstuffs is inevitable, thus preservative measures must be directed to minimise the level of contamination that reaches the product in the early steps of the food chain. Hygienic control in processing and food handling, and techniques like aseptic packaging of thermally processed foods, works towards this objective (Gould, 1996a). However, if the fungus gains access to the product the objective turns to controlling its activity and growth on the food itself. For inactivating or inhibiting fungal growth in foods, several physical, chemical and biological measures can be taken.

(a) Physical Methods

Physical preservation methods subject the food to measures which adversely affects fungal growth. The best known are heat treatments (sterilisation and pasteurisation), refrigeration (cooling and freezing), dehydration (drying), and irradiation. Although several irradiation techniques have been proven to be useful measures to inactivate and control microbial food-spoilage, their bad publicity has limited their use in the food industry. At present, heat and dehydration are among the most used physical methods for food preservation.

Heat treatments like pasteurisation and sterilisation inactivate or destroy microbial cells. However, not all microorganisms are equally susceptible to high temperatures. Furthermore, microbial heat resistance is influenced by many factors such as pH, water

content, number of organisms initially present or the nutritional status of the food. Preservation of foods by drying is based on the fact that microorganisms and enzymes need water for their correct functioning. Lowering moisture content of the foodstuff by adding solutes or by efficient drying, results in food-spoilage and food-poisoning microorganisms being inhibited (Jay, 2000).

New physical techniques are continuously being developed in response to the increasing demand for fresh and mild processed foods: high-pressure processing, pulse electric fields, thermoultrasonication or aseptic packaging have all been successfully applied to a number of foodstuffs.

(b) Chemical Methods

Chemical methods are characterised by the addition of a chemical substance that inhibits the development of microorganisms. These substances are known as antimicrobial preservatives and are treated in more detail in section 1.2.2.

(c) Biological Methods

In some cases, harmless microorganism cultures known as protective cultures (Jay, 2000) are added to the foods. These cultures have an inhibitory effect on undesirable spoilage microorganisms. For example, growth and toxin production by *P.verrucosum* has been shown to be lower in the presence of the food grade yeast *Saccharomyces cerevisiae* in wheat substrates (Petersson *et al.*, 1998).

1.2.2 Antimicrobial agents: an overview

The most common way to prevent or control mould growth in foodstuffs is by the use of antimicrobial agents. Antimicrobial agents are chemical substances that when added to foods tend to prevent or retard microbiological food spoilage. These substances are generally known as food preservatives.

Food preservative, however, is a wider term that refers to all chemical products used to control any kind of food deterioration, either physical, chemical or microbiological. Furthermore, salt, sugars, vinegar, spices and essential oils, recently studied for food preservation because of their antimicrobial properties, are not included in this definition (De Boer and Nielsen, 1995).

In practice, antimicrobial agents have been frequently distinguished into two main groups whether they inhibit growth (fungistatic or bacteriostatic effect) or destroy the spoilage microorganism (fungicidal or bactericidal). Although this distinction is widely accepted and used by most scientists, some authors find it unjustifiable on the basis that these two groups just differ from each other in the death rate of the microorganisms (Lück and Jager, 1997). In fact, they maintain that the long term effect of an added preservative in a food product is either to kill the microorganisms or allow them to grow, with the dosage the most important factor that determines its effectiveness. Ideally, any antimicrobial substance should inhibit microorganisms in their initial lag phase of growth and not in the exponential log phase, since in the latter case the necessary dosages of the agent would be too high (Smid and Gorris, 1999).

(a) Effect on microorganisms

In general, all antimicrobial agents as well as all the measures taken to preserve food products tend to temporarily or permanently disturb the homeostasis, i.e. internal cell stability, of the living organisms. When this occurs, the organism uses up all the energy to overcome this disruption so it can not multiply, remaining in the lag-phase or even dying, before homeostasis is re-established (Leistner, 2000).

The effect of antimicrobials on the cell homeostasis of spoilage microorganisms is based on a multiplicity of individual influences. Some antimicrobials, like salts or sugars that reduce the a_w of the substrate, do not affect the cell directly but create an environment in which microbial growth is inhibited. For those substances that actually affect the cell function, Lück and Jager (1997) summarised their action into the following effects:

- Influence on the DNA,
- Effects on protein synthesis,
- Influence on enzyme activities,
- Influence on cell membrane and cell wall stability,
- Influence on transport mechanisms for nutrients.

It is increasingly being assumed that most preservatives act on the cell wall and cell membrane, affecting their structure and the transport mechanisms of nutrients into the cell (Lück and Jager, 1997). For example, benzoic acid acts directly on both, the cell walls and by inhibition of citrate cycle enzymes and of enzymes involved in oxidative phosphorylation (Belitz and Grosch, 1999).

(b) Selecting the suitable antimicrobial

To achieve maximum effectiveness when selecting a food antimicrobial agent, several factors must be taken into consideration.

(i) Antimicrobial spectrum of the compound

No antimicrobial agent has the same effect against all spoilage microorganisms. While some chemicals such as the antibiotic natamycin are mainly active against fungi (De Boer and Nielsen, 1995), others like benzoic or sorbic acids, are effective against both filamentous fungi and yeasts and to a lesser extend, bacteria (Lück and Jager, 1997). The knowledge of the antimicrobial spectrum of a certain compound and the bio-burden of the food product will allow a more effective use of the antimicrobial agent (Davison and Juneja, 1990).

(ii) Chemical and physical properties of the antimicrobial agent and the food product

Chemical antimicrobial substances are dependent on physico-chemical properties of the substrate and environment. Hydrophilic and lipophilic properties are important factors

to consider. Antimicrobial products require a certain degree of both water solubility and lipid solubility. Antimicrobials should be water soluble, since microorganisms mostly grow in the aqueous phase of the foods, and they should have lipophilic characteristics because, as previously mentioned, most antimicrobial agents act on, or through, the hydrophobic cell membrane of the microorganisms (De Boer and Nielsen, 1995).

The nutritional status of the food is also important. Food products rich in nutrients and vitamins for example, foster the growth of microorganisms so they will likely overcome any unfavourable condition for their growth.

(iii) Initial microbiological quality of the food product and conditions of its storage

None of the antimicrobial agents commonly used in food preservation is able to preserve a highly contaminated product (Davison and Juneja, 1990). As Lück and Jager (1997) pointed out preservatives cannot be used to compensate for poor factory hygiene and, in fact, for the concentrations at which most preservatives are used, this would not even be possible.

(iv) Safety and legal considerations

Antimicrobials and their degraded products have to be toxicologically safe. On this basis, all antimicrobial compounds as well as all food additives are legislatively prohibited unless they have been expressly approved at a certain permissible maximum quantity and field of use. Table 1.1 shows the ADI values (Acceptable Daily Intake) established by JECFA (Join Expert committee of the FAO/WHO) for some of the most common antimicrobial agents used in the food industry.

In some countries as in the UK, stipulation for preservatives are frequently contained in specific regulations while in other countries like France, these substances are covered by the regulations on individual food products. Furthermore, the number and type of antimicrobials permitted in a certain foodstuff varies between countries. Table 1.2 gives a list of preservatives permitted for use in foods within the European Union.

Table 1.1 ADI values of some antimicrobials (Davison and Juneja, 1990, De Böer and Nielsen, 1995; Lück and Jager, 1997)

ANTIMICROBIALS	ADI (mg/Kg body weight)
➢ Acetic acid and its salts	no limit
➢ Benzoic acid and its salts	0-5
➢ *p*-Hydroxybenzoic acid esters	0-10
➢ Nitrite	0-0.2
➢ Sulfur dioxide, sulphites	0-0.7
➢ Natamycin	0-0.3
➢ Nisin	0-33000 (units)
➢ *o*-phenylphenol	0-0.2
➢ Propionic acid and its salts	no limit
➢ Sodium diacetate	0-15
➢ Sodium and potassium nitrate	0-5
➢ Sorbic acid and its salts	0-25
➢ Sodium chloride	1100-3300

Table 1.2 Preservatives permitted for use in foods within the European Union (Lück and Jager, 1997)

E200	Sorbic acid	E224	Potassium metabisulfite	E261	Potassium acetate
E303	Potassium sorbate	E226	Calcium sulfite	E262	Sodium acetate
E203	Calcium sorbate	E227	Calcium hydrogen sulfite	E263	Calcium acetate
E210	Benzoic acid	E228	Potassium hydrogen sulfite	E270	Lactic acid
E211	Sodium benzoate	E230	Biphenil	E280	Propionic acid
E212	Potassium benzoate	E231	Ortophenil phenol	E281	Sodium propionate
E213	Calcium benzoate	E232	Sodium ortophenil phenolate	E282	Calcium propionate
E214	Ethyl *p*-hyroxybenzoate	E233	Thiabendazol	E283	Potassium propionate
E215	Sodium ethyl *p*-hydroxybenzoate	E234	Nisin	E284	Boric acid
E216	Propyl *p*-hydroxybenzoate	E235	Natamycin	E285	Sodium tetraborate
E217	Sodium propyl *p*-hydroxybenzoate	E239	Hexamethylene tetramine	E290	Carbon dioxide
E218	methyl *p*-hydroxybenzoate	E242	Dimethyl dicarbonate	E941	Nitrogen
E219	Sodium methyl *p*-hydroxybenzoate	E249	Potassium nitrite	E1105	Lysozyme
E220	Sulfur dioxide	E250	Acetic acid		
E221	Sodium sulfite	E251	Sodium nitrate		
E222	Sodium bisulfite	E252	Potassium nitrate		

(v) Other factors

Many other factors have to be considered when selecting an antimicrobial agent: the preservative, for example, should cause no disruption of desirable microbiological processes occurring in the food, e.g. yeast fermentation in baked goods; they should not react with the food components and should minimally affect the sensory characteristics of the food. Finally, the preservative should be economically feasible.

1.2.3 Existing chemical antimicrobials

Generally, physical methods are not sufficient to control food spoilage, so some antimicrobial agents often need to be added. A wide number of chemical substances are commonly used to preserve food commodities from microbiological spoilage. Nitrites, parabens, sulfites, sodium chloride, and organic acids are some of the chemicals most generally used.

Nitrites are used primarily to preserve the red colour of meat and to inhibit the growth and toxin production of *Clostridium botulinum* in cured meats, but has also been effectively used to preserve fish and cheese products. Their effect particularly at low pH is exclusively against bacteria (Davison and Juneja, 1990). These chemicals however, are being restricted because of the possible formation of nitrosamines within the food product, which exerts powerful carcinogenic activities (Belitz and Grosch, 1999).

Sulfites are primarily added to fruit and vegetable products to control bacteria, fermentation and spoilage yeasts, and moulds (Davison and Juneja, 1990; Eberlein-König *et al.*, 1993). Moulds such as *Botrytis* on grapes for example, can be controlled by periodic gassing with SO_2 (Jay, 2000). SO_2 is also used in certain foods because of its antioxidant properties.

Sodium chloride (NaCl) has been used as a food preservative since ancient times. Although today it is still considered as an antimicrobial agent for foodstuffs, it is generally used together with other processing methods such as canning and

pasteurisation (Sofos, 1983). Salt acts by reducing the a_w of the substrate and thus creating unfavourable conditions for microbial growth. Sugars, such as sucrose, exert their preservative action in essentially the same way as salt but, generally six times more sucrose than NaCl is needed to give the same degree of inhibition (Jay, 2000).

Pesticides such as dichlorvos and thiabendazole have been examined for use in food preservation. Dichlorvos, an organophosphate insecticide shows a potent antimycotic and antitoxigenic activity at relatively low levels (Radic *et al.*, 1991). However, the use of pesticides on crops and food commodities is also continuously being restricted, moving towards their complete banning.

Parabens i.e. esters of 4-hydroxybenzoic acid (PHB), were initially synthesised as a possible replacement of existing preservatives like salicylic and benzoic acids, effective only in the highly acid pH range.

Although Lück and Jager (1997) maintain that one of the most important characteristics of parabens, together with a much higher antimicrobial action to that of phenols and organic acids, is their pH independent activity, some studies have reported a slight influence of pH on their activity (Thomson *et al.*, 1993). Nevertheless, due to their high pKa value (8.5), parabens are chemical preservatives effective over a wider range of pH (3-8). Antimicrobial activity of parabens is related to the length of the ester group of the molecule.

As additives, parabens are applied as alkali solutions or as ethanol or propyl glycol solutions in fillings for bakery products, fruit juices, marmalades, syrups, preserves, olives and pickled vegetables (Belitz and Grosch, 1999). However, few studies have examined their potential for controlling spoilage moulds in bakery products.

Synthetic antioxidants Together with parabens, other phenolic-derived antioxidants have been screened for their possible antimicrobial efficacy. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and 2-tert-butylhydroxybenzoic (TBHQ) are among them. Kubo *et al.* (2001) comparing the

antifungal activity of three gallates: propyl (C3), octyl (C8) and dodecyl (C12) found that only octyl gallate was the only active compound against four different fungal genera with a MIC of 25ppm.

However, little information is found in the literature relating to the antimicrobial activity of these compounds compared to that found for other preservatives. The majority of these compounds are currently permitted for use as antioxidant additives in food and cosmetic products but no regulations exist to date for their use as antimicrobials in food.

Organic acids are the main group of antimicrobials used in food preservation especially against mould spoilage. Organic acids can be divided into two main different groups depending on the effect they exert: strong acids and weak acids.

(i) Strong acids

Strong acids such as malic, formic and tartaric acids, lower the external pH but do not penetrate the cell membrane. These types of organic acids are more effective against yeast and bacteria than against fungi. Nevertheless, some derived products like the sodium salt of acetic acid has been reported to be effective in inhibiting cheese spread spoilage moulds (Doores, 1983) and some *Aspergillus* and *Penicillium* spp. at a dose of 0.1-0.5% (Glabe and Maryanski, 1981). The effect of low external pH on microbial growth has been suggested to be due to either inactivation of one or more enzyme activities at the outer layers of the cell or a reduction of transport systems for essential ions and nutrients (Rahman, 1999).

(ii) weak acids

Weak acids are lipophilic acids that penetrate the cell membrane in its undissociated from. When the undissociated acid enters the cell a higher pH environment is encountered, the molecule dissociates resulting in the release of charged anions and protons which cannot cross the plasma membrane.

The primary effect of weak organic acids is, then, to disturb pH homeostasis by lowering the cytoplasmic pH. Nevertheless, the undissociated acid may have specific effects on the cell metabolism that may amplify the effects of the weak acid. In fact, microbial growth inhibition by weak acids has been proposed to be due to a number of actions such as membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis, and the accumulation of toxic anions (Brul and Coote, 1999; Jay, 2000).

The high solubility, low taste and toxicity of weak organic acids make them highly suitable to be used in food preservation (Ray and Bullerman, 1982; Davison and Juneja, 1990). pH of the environment and solubility of the acid often determines the foods in which these acids may be effectively used (Ray and Bullerman, 1983). Because of their low pKa value (4.19-4.87), these substances are effective antimicrobials in low pH substrates since acidic environments favours the undissociated form of the molecule which is freely permeable across the membrane. Table 1.3 shows the % of undissociated form of some organic acids at different pH values.

Sorbic, benzoic and propionic acids and their salts, known as sorbates, benzoates and propionates, are the most widely used antifungal agents in foodstuffs. These acids are Generally Recognised As Safe (GRAS) (Liewen and Marth, 1985; Binstok *at al.*, 1998). Sorbic acid, for example, has a half-life in the body of about 40-110 min and in normal conditions it is completely oxidised to CO_2 and H_2O (Liewen and Marth, 1985). However, because of their higher water solubility and easier handling than their acids, potassium, sodium or calcium salts are the forms more commonly used.

Sorbates.

Sorbates have been used as effective antifungal agents since 1945. Sorbic acid and its salts are among the most thoroughly investigated of all preservatives. Their main use is in preservation of food, animal feed, cosmetic and pharmaceutical products (Liewen and Marth, 1985). Table 1.4 shows minimum inhibitory concentrations of sorbic acid for some fungal species measured on nutritive media test.

Table 1.3 Percentage of undissociated form of some organic acids at different pH-values (De Boer and Nielsen, 1995).

Percentage of undisociated form (%)

		pH						
Organic acid	**pKa**	**2.5**	**3.5**	**4.5**	**5.0**	**5.5**	**6.0**	**7.0**
Acetic acid	**4.74**	99	95	63	35	14	5.2	0.55
Citric acid	**3.13**	81	30	4.1	1.3	0.4	0.13	0.01
Benzoic acid	**4.19**	98	83	33	13	4.7	1.5	0.15
Propionic acid	**4.87**	100	96	70	43	19	6.9	0.74
Sorbic acid	**4.76**	99	95	65	37	15	5.4	0.57

Table 1.4 Minimum Inhibitory concentration (MIC) of sorbic acid against several mould species (Lück and Jager, 1997).

Fungal species	**pH value**	**MIC (ppm)**
Rhizopus species	3.6	1200
Penicillium species	3.5-5.7	200-1000
Penicillium digitatum	4.0	200-1000
Penicillium glaucum	3.0	1000-2500
Aspergillus species	3.3-5.7	200-1000
Aspergillus flavus	-	1000
Aspergillus niger	2.5-4.0	1000-5000
Fusarium species	3.0	1000
Cladosporium species	5.0-7.0	1000-3000

The effectiveness of sorbates on various strains of microorganisms has been investigated in a number of general studies (Liewen and Marth, 1984; Davison and Juneja, 1990; Sofos 1992). There are, however, some moulds belonging to the genus *Penicillium* particularly *Penicillium roquefortii* (Belizt and Crosch, 1999), that can degrade sorbic acid, through a decarboxylation reaction which is carried out in the fungal mycelium, and produces 1,3-pentadiene, which possess a very strong hydrocarbon-like odour (Finol *et al.*, 1981). This degradation may occur when sorbic acid is present in sub-lethal concentrations and the microbe counts are high (Lück and Jager, 1997).

Sorbic acid, potassium and calcium sorbate are permitted in all countries of the world for preservation of many foods such as cheese, meat products, fish products, fruits, vegetables, drinks or baked goods. In the meat industry, for example, sorbates are common inhibitors of mould growth in sausages. The use of sorbates in combination with nitrites has been recommended in order to decrease the requirements for nitrite addition to foods (Binstok *et al.*, 1998).

The resistance of the lactic acid bacteria to sorbate, specially at pH 4.5 or above, permits its use as an antifungal agent in products that undergo lactic fermentation (Jay, 2000). The type of food in which the use of sorbates is permitted can vary between different countries In general terms, the maximum permissible quantities are ranged between 0.1 and 0.3% (Lück and Jager, 1997).

Potassium sorbate is the salt most commonly used. It was specifically developed to prepare the aqueous stock solution needed for dip and spray applications. Although potassium sorbate is 75% less effective than the acid (Ray and Bullerman, 1982; Sofos, 1989) it is less corrosive and more soluble in water.

Potassium sorbate shows various potential inhibition mechanisms that affects intracellular and membrane enzymes which are essential to the life of microorganisms (Sofos, 1989). The primary inhibitory action of sorbates is against yeasts and moulds. This salt, for instance, has been proved to be an effective fungistatic agent against

toxigenic moulds at levels from 0.10 to 0.15% (Ray and Bullerman, 1982). The activity against bacteria appears to be selective (Kubo and Lee, 1998).

Benzoates and Propionates

Benzoates and propionates are also used in food preservation although to a lesser extend than sorbates. Benzoic acid has the lowest pK_a value of all three acids. At pH 4.5 only the 33% of the molecule is present in its effective undissociated from (see Table 1.3, p. 19).

Benzoic acid is commonly used as its sodium salt, sodium benzoate, and its application in food preservation is restricted to more highly acidic products such as certain fruits and vegetables (Lück and Jager, 1997).

Propionic acid has a weaker antimicrobial action and thus it has to be employed in relatively high dosages in order to preserve foodstuffs. Propionic acid is found naturally where propionic acid fermentation occurs e.g. in emmental cheese in which it is present in up to 1% (Belitz and Grosch, 1999). The use of propionates has been directed against moulds but it has also been proven to be a very effective inhibitor of the bacterium *Bacillus subtilis* which causes rope in bread (Davison and Juneja, 1990). Table 1.5 reviews some characteristics of propionic, benzoic and sorbic acid.

1.2.4 Natural antimicrobials

Nature contains many different types of antimicrobial compounds which play an important role in the defense or competition systems of microorganism, insects, animals or plants. These substances may be effectively applied in food preservation to reduce chemical preservatives in foods in response to consumer demand. Although in the last few years many studies have been carried out in this area, the use of so-called natural antimicrobials in food commodities is still restricted to a small group of compounds.

Table 1.5 Some characteristics of propionic acid, benzoic acid and sorbic acid.

	PROPIONIC ACID	SORBIC ACID	BENZOIC ACID
✶**COMMERCIALLY AVAILABLE FORMS**	Propionic acid Sodium propionate Calcium propionate	Sorbic acid Potassium sorbate Calcium sorbate	Benzoic acid Sodium benzoate
✶**FORMULA**	CH_3CH_2COOH	CH_3-CH=CH-CH=CH-COOOH	C_6H_5COOH
✶**PROPERTIES**	-Colorless -Disagreeable pugnent odor -Unlimitted miscibility in water	-Faint odor -Sour taste - 0.16% water solubility	-Strong odor -0.34% water solubility
✶**TOXICITY (LD_{50}, rats/oral)**	2.6 g/Kg body weight	7.4-10.5 g/kg weight	1.7-3.7 g/Kg body weight
✶**APPLICATIONS**	Cheese Baked goods	Margarine Mayonnaise Cheese Meat (sausages, cured meat) Fresh fish Fermented vegetables products Vegetables pickled in vinegar Dried prunes Fruit pulps Fruit juices Wine Baked goods Confectionery	Margarine Pickled vegetables Acid fruit products Fruit juices Soft drinks
✶**LIMIT PERMITTED**	0.3%	0.1-0.2%	0.15-0.25%

Antimicrobials derived from plants and microorganisms are the most feasible substitutes for chemical food antimicrobials in either practical and ethical aspects and legislation (Smid and Gorris, 1999). Figure 1.1 represents the main groups of natural antimicrobials found in nature with possible use for food spoilage control.

(a) Microbe-derived antimicrobials

Microorganisms produce several compounds that control the growth of other microorganism present in their environment. Antimicrobials of microbial–origin used in food commodities have been named as 'biopreservatives'. It has been demonstrated that some fungal metabolites can inhibit the growth of other fungal species. Paster *et al.* (1992) found that media previously colonised by *Aspergillus niger* were effective at inhibiting growth of *A.flavus* and *A. ochraceus* when the fungi were grown in a nutritive media where *A.niger* previously grew. Nevertheless, when talking about 'biopreservatives' it is almost always exclusively refers to those antimicrobials produced by lactic acid bacteria (LAB).

Lactic acid bacteria produce GRAS antibacterial compounds with a broad antimicrobial spectrum, like organic acids or hydrogen peroxide, and compounds with a narrower spectrum called bacteriocins (Smid and Gorris, 1999). Bacteriocins are peptide antibiotics primarily lethal to other strains and species of bacteria (Konings *et al.*, 2000). LABs produce many different types of bacteriocins with possible application for food preservation. Nisin is now the only bacteriocin legally approved for use as a food additive (Gänzle *et al.*, 1999). This bacteriocin is successfully used in canned foods such as soups or vegetables, together with heating processes to control heat-resistant bacterial spores (Gorris and Bennik, 1994). In some fermented products such as meat, dairy products or vegetables, lactic acid bacteria can be added directly. In these products the bacteria gives the food the desired sensory and organoleptic properties while, at the same time, the *in situ* growing LAB produces many different types of antimicrobial substances that inhibits a wide range of microorganisms that can deteriorate the product. In this case, LABs are known as 'protective cultures'.

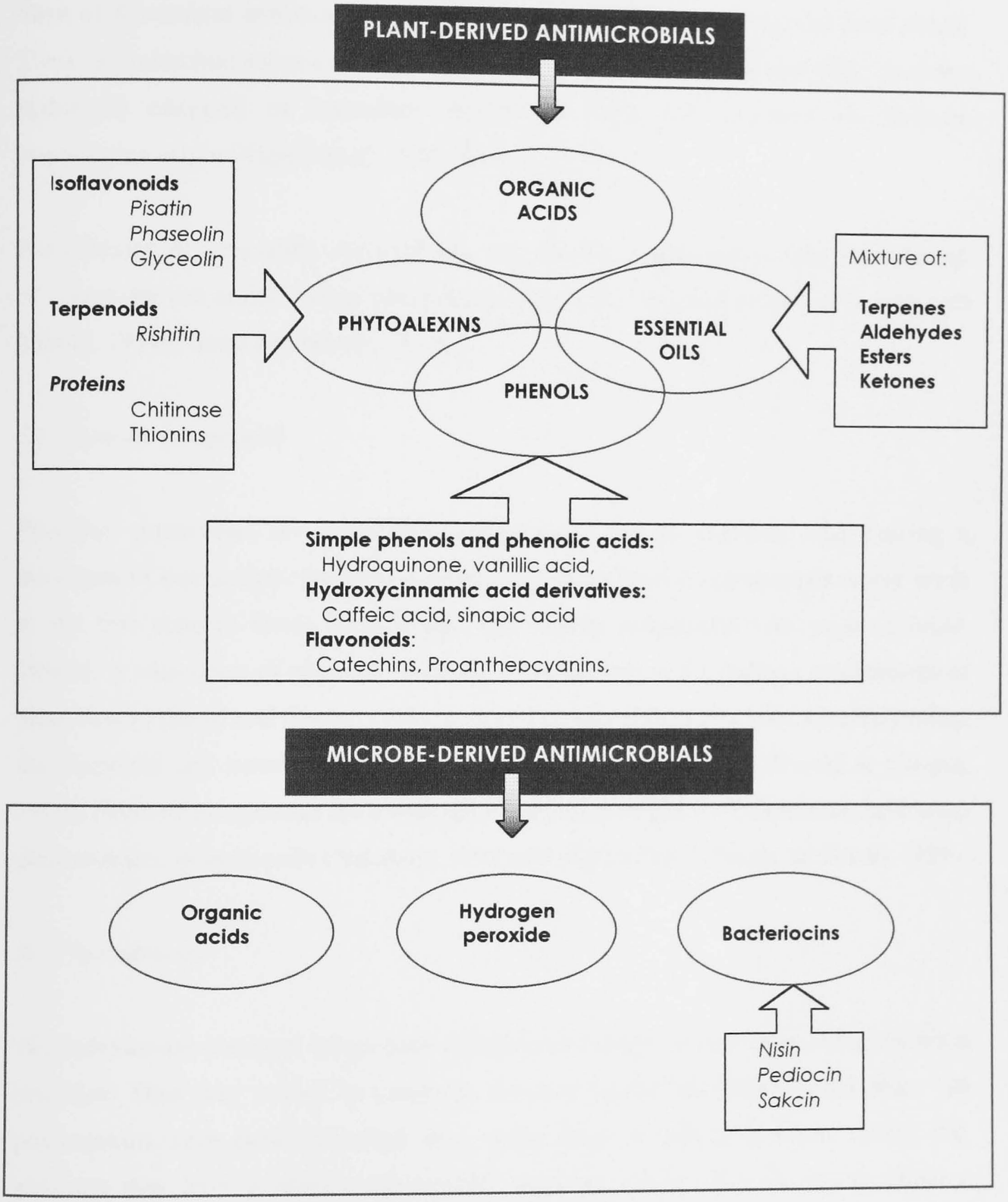


Figure 1.1 Antimicrobial agents found in natural commodities.

(b) Plant-derived antimicrobials

Most of the natural antimicrobials used in food commodities are derived from plants. These antimicrobial substances are known as 'green chemicals' and are either proteins, hydrolytic enzymes or secondary metabolites from the terpenoid or phenolic biosynthesis origin (Vigers *et al.*, 1991; Bowles, 1995).

Phytoalexins, organic acids, essential oils and phenolic compounds are the main groups of plant-derived antimicrobial compounds considered for food preservation purposes (Gould, 1996b; Smid and Norris, 1999).

(a) Phenolic compounds

Phenolic compounds are molecules characterised by an aromatic ring bearing a minimum of one or more hydroxy substituents. These types of compounds rarely occur in the free state in living plant tissues but usually conjugated with sugars (Gould, 1996b). A vast range of phenolics compounds contribute to the defense mechanisms of plant tissues (Smid and Gorris, 1999). It is known that these molecules adversely affect the microbial cell membranes or inhibit the germination of spores (Russel & Chopra, 1990). Phenolic compounds are a wide group of antimicrobials which also include some plant extracts, essential oils (Nakatani, 1994) and phytoalexins (Smid and Gorris, 1999)

(b) Phytoalexins

Phytoalexins are chemical compounds synthesized in plants as a response of a microbial infection. Thus they cannot be generally found in unaffected tissues. More than 100 phytoalexins have been identified in a wide range of plants (Nychas, 1995) and, although they have a broad antimicrobial spectrum, their action is mainly directed against fungi (Nychas, 1995; Smid and Gorris, 1999).

Isoflavonoids are among the most important chemical type of phytoalexins, and their possible application in microbial spoilage control of some foodstuffs has been studied

(Weidenbörner and Jha, 1993). Nevertheless, the actual use of these compounds in food preservation is limited, possibly due to the fact that, in general, phytoalexins show an adequate antimicrobial effect at relatively high concentrations (Gould, 1996b).

(c) Essential oils

In the last few years great interest has emerged in the possible use of plant extracts and essential oils for food preservation. Essential oils are mostly derived from spices, i.e. dried aromatic products, obtained from different parts of the plant such as leaves (e.g. rosemary, sage), flowers (e.g. clove), bulbs (e.g. garlic, onion) or fruits (e.g. pepper, cardamon) (Shelef, 1983).

Plant and plant extracts have been used historically for many years in medicine and food preservation. Extracts and essential oils of many of these plants are now being screened for their antimicrobial effectiveness. For example, several Indian plants have been reported to be active antimicrobial agents against several post-harvest spoilage bacteria (Farag et al., 1989; Sinha, 1990; Sinha and Gulati, 1990; Saxena and Sharma, 1999; Deana and Thoppil, 2000) and fungi (Dwivedi and Dubey, 1993; Bourrel and Perineau, 1993; Saxena and Mathela, 1996; Rana and Taneja, 1997; Flamini *et al.*, 1999; Singh and Tripathi, 1999; Deana and Thoppil, 2000; Saxena and Sharma, 1999). *Aspergillus flavus*, one of the most toxigenic foodborne fungi, has been found to be inhibited by some of these plant derivatives. Dwivedy and Dubey (1993), for instance, studying the antifungal activity of several umbelliferous plant essential oils against *Aspergillus* species found an important fungistatic effect of *Trachyspermum* seed essential oil at relatively low concentrations (< 500ppm).

The number and type of plant essential oils tested for food preservation is increasing. Extracts and essential oils of very well known plants such as oregano, thyme, basil, garlic, onion, clove and cinnamon have been reported as some with the greatest antimicrobial effectiveness (Paster *et al.*, 1995; Özcan, 1998; Basilico and Basilico, 1999; Lachowicz *et al.*, 1998; Wan *et al.*, 1998; Pai and Platt, 1995; Yin and Tsao, 1999; Yin and Cheng, 1998; Bigrami *et al.*, 1992).

More specifically, Azzou and Bullerman (1982) stabilised clove and cinnamon as the strongest antifungal agents against *Penicillium* and *Aspergillus* species. However, most of these studies did not study the effect of different environmental factors on the essential oil effectiveness. Oregano and thyme are two of the plant essential oils more extensively considered for food preservation. Paster *et al.* (1995) studied the effectiveness of oregano and thyme essential oils in control of post-harvested grain spoilage by *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger*. They found that the very low dosage of 2 µl/L (in the vapour phase) of oregano oil applied as a fumigant, completely inhibited the mycelial growth of all three species during the 7 days of the experiment. Thyme essential oil was less effective, permitting mycelial growth at dosages above 4 μl/L.

When the oil is incorporated directly into the substrate, the effectiveness appears to be lower. In fact, up to 1000 ppm of oregano essential oil was necessary to completely inhibit growth of *A.flavus* in liquid media for 21 days (Basilico and Basilico, 1999) and 100 ppm to completely inhibit growth of *Aspergillus niger* and several *Penicillium* species over a period of 6 days. However, this fungistatic activity of oregano essential oil contradicts the results reported by Salmeron *et al.* (1990) who found a stimulation effect on the growth of the same *Aspergillus* species when the plant of thyme and oregano were incorporated into nutritive media.

Chemically, plant essential oils consists of a mixture of esters, aldehydes, ketones and terpens. Although several studies have been carried out on the inhibitory effect of essential oil components (Lachowicz *et al.*, 1998; Saxena and Mathela, 1996; Consentino *et al.*, 1999; Mahmoud, 1994; Sinha and Gulati, 1990; Bigrami *et al.*, 1992), the role of these components in the antimicrobial activity of the oil is not clear. Various oil components may act synergistically in the antimicrobial activity while others can stimulate fungal spore germination (French, 1985).

Phenolic compounds are probably the major type of ESO components (Sinha, 1990; Smid and Norris, 1999). Figure 1.2 shows the chemical structure of some active antimicrobial essential oil constituents.

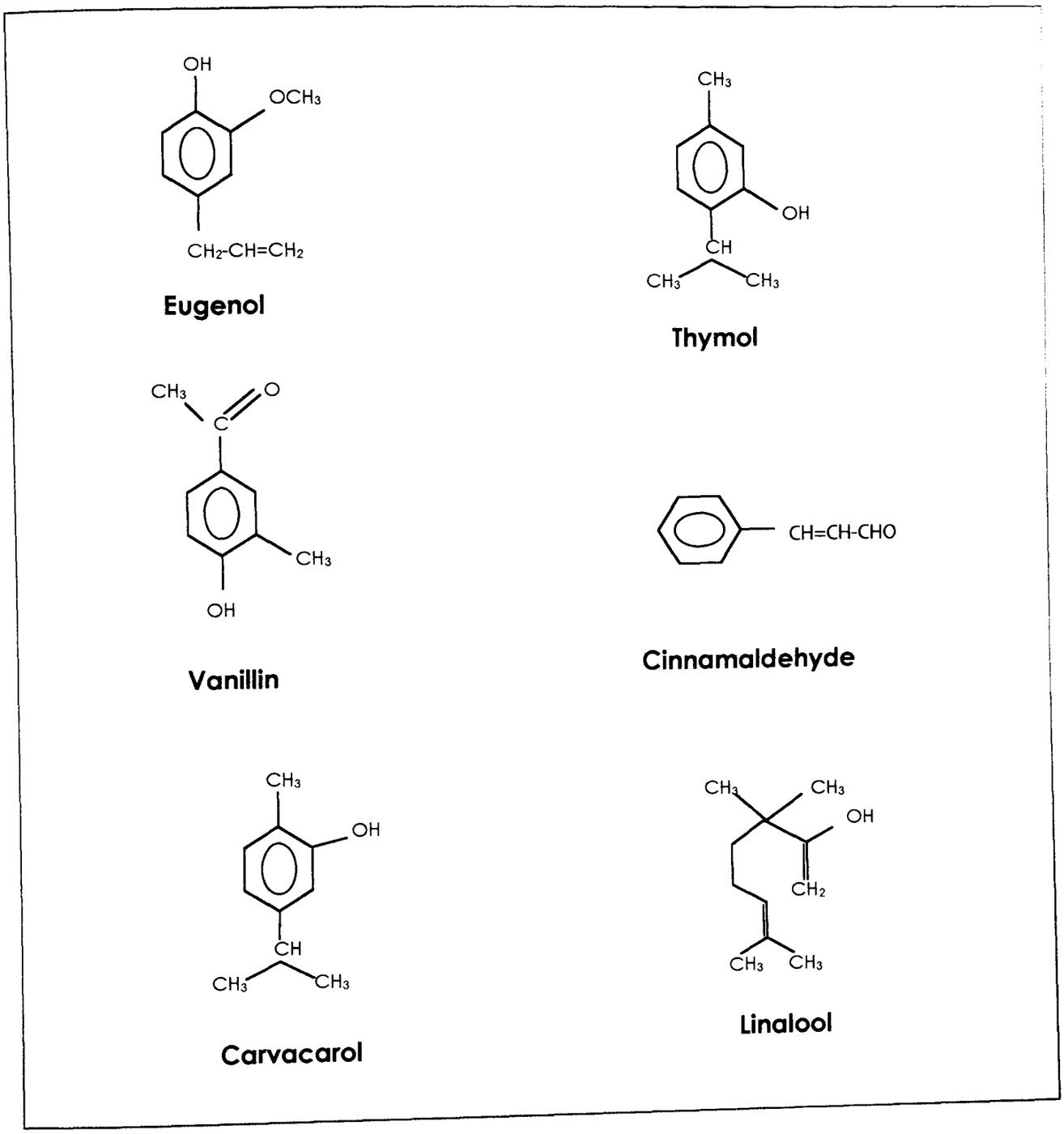


Figure 1.2 Chemical structure of some essential oil components.

Thymol from thyme and oregano, cinnamaldehyde from cinnamon and eugenol from cloves are among molecules with a wider range of antimicrobial effectiveness (Consentino *et al.*,1999; Gould, 1996b). Cinnamaldehyde exerts potent antifungal activity against several food-associated fungi belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera (Smid and Gorris, 1999). Studying the effect of 20 different essential oil constituents on the growth and aflatoxin production of *Aspergillus flavus*, Mahmoud (1994) established the MIC (Minimum Inhibitory Concentration) for thymol and cinnamaldehyde at 250ppm and 200ppm respectively and found complete inhibition of its growth and toxin production at the dosage of 1000 ppm.

On the other hand, Lachowicz and co-workers (1998) comparing the inhibitory effect of sweet basil and its main constituents (methyl chavicol and linalool) on the growth of some bacteria and fungi, observed lesser antimicrobial activity of linalool-methyl chavicol combinations to that obtained with basil essential oil, indicating that other less predominant(s) components are most likely contributing into the antimycotic activity of the oil.

Most of the work done on the antimicrobial efficacy of plant extracts and essential oils has been carried out in nutritive media. When the essential oil is tested in the food product, less efficacy has been observed, probably due to the fact that specific components of the food product such as proteins or fats can bind essential oil components, inactivating them (McNeil and Schmidt, 1993; Smid and Gorris, 1999).

The use of essential oils in foods also has other limitations; the doses at which these substances exert their antimicrobial activity, for example, are relatively high, thus their typical odour is also noticeable. In some products however, this flavour is desirable. In tomato-based products, for example, the addition of basil can be used both as a flavouring and as an antifungal agent (Lachowicz *et al.*, 1998). However, very few studies on the effect of interacting environmental factors such as pH, a_w and temperature on essential oil effectiveness against spoilage moulds of bakery products are found in the literature.

1.2.5 Combined methods in food preservation

As has been pointed out in the last sections, microbial stability of food products are dependent on a wide number of factors. The knowledge of these individual factors has allowed the development of the different techniques commonly in use for food preservation. However, these techniques are rarely used alone since the severity of the treatment necessary to control microbiological spoilage will adversely affect the sensory and nutritional quality of the food product.

The combination of different preservation procedures in order to avoid microbial growth in food commodities has been named by Leistner as "hurdle technology" (1985). However, this term is basically applied for combinations of preservatives and physical measures (Leistner, 1985; Leistner, 1992; Leistner, 2000). For instance, Akopomedaye and Ejechi (1998) found that several *Aspergillus* species were more effectively inhibited when heat and ginger extract were used in combination than when the preservative factors were used alone.

Different preservatives can also be used in combination with one another. This practice may lead to a broader spectrum of action of the preservative, an increase of antimicrobial activity, and may enable the use of lower concentrations (Lück and Jager, 1997). The antifungal activity of potassium sorbate, for example, has been enhanced by other antimicrobial compounds like vanillin (Matamoros Leon *et al.*, 1999) and polyglodial (Kubo and Lee, 1998).

1.3 BREAD

1.3.1 Situation of the bakery sector

Bread is defined by the Bread and Flour Regulations (1998) as a "food of any size, shape or form which (a) is usually known as bread, and (b) consists of a dough made from flour and water, with or without other ingredients, which has been fermented by yeast or otherwise leavened and subsequently baked or partly baked".

Bread is one of the most important dietary staples in the world. In the UK annual bread consumption was estimated at 41.5 Kg per person (see Legan, 1993). In the UK typical production consists of up to 10.000 loaves an hour operating 24 hours a day. A total of 43 million loaves of wrapped bread are delivered weekly of which 99% is nationally produced (Casdagli, 2000).

There are many different types of bread all over the world. In many countries as in the UK, bread is light in texture, almost exclusively made of wheat flour, with a pH between 5.4-6.0 and leavened by a yeast fermentation. In some other countries such as Germany and many eastern European countries, rye breads made by a sourdough fermentation processes and of much lower pH (3.5-4.8) are popular (Legan, 1993).

Bread is a highly perishable food product. The three most common forms of bread deterioration are staling, moisture loss and microbiological spoilage (Seiler, 1984). It is not clear what percentage (%) of economic losses in the bakery industry can be attributed to mould spoilage. Assuming only 1% losses, moulds could result in losses of £20 million in the UK every year (Legan, 1993).

1.3.2 Mould spoilage

From a microbiological point of view the most important factor common to different breads is a high moisture content and thus their a_w (about 0.94-0.97) (Legan, 1993) This characteristic makes bread highly susceptible to microbial attack. The common sliced and wrapped bread is more likely to be spoiled by moulds than unwrapped bread. In this type of bread, slicing provides more moist surfaces for moulds to grow on and wrapping prevents moisture lost.

Mould contamination of bread (more than 90% of the total contamination in wheat sliced bread) most likely occurs during cooling, slicing or wrapping operations, as the high cooking temperatures are enough to eliminate possible previous contamination (Legan, 1993; Roesler and Ballenger, 1996). Contamination then, comes from the bakery equipment, environment, from the flour dust spread throughout the bakery which

contains many fungal spores (Gemeinhard and Bergmann, 1977; Spicher, 1980; Ooraikul, 1987; Legan and Voysey, 1991) but mainly from mould spores introduce in the bakery from the outside (Yassin and Wheals, 1992).

Many fungal species are implicated in spoilage of bread and bakery products but the most commonly found belong to the genus *Penicillium* (Patterson *et al.*, 1983; Legan, 1993). In yeast-raised wheat breads a wide range of spoilage moulds including *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucorales* and *Neurospora* have been found (Legan, 1993; Viljoen and Von Holy, 1997).

Although *Penicillium* spp are the principal contaminants in different types of bread, their incidence is affected by the storage temperature. Some authors found a 50% decrease of *Penicillium* spoilage incidence when the temperature was raised from 4 to 22-24°C. In warmer climates the presence of *Aspergillus* spp. becomes more prominent (Legan, 1993).

But moulds are not the only bread contaminants. Rope is a real problem in bread caused mainly by certain strains of *Bacillus subtilis.* When the heat-resistant spores of the organism survive baking and multiply in the bread crumb they cause the development of a characteristic fruity odour followed by a decomposition of the crumb which becomes sticky and discoloured (Seiler, 1984; Thomson *et al*, 1993). Contrary to mould spoilage, bread raw materials (e.g. yeast, flour, bread crumbs) have been identified as the main source of potential spoilage bacteria (Viljoen and Von Holy, 1997).

In addition to economic losses associated with bread spoilage, possible mycotoxin production is also of great concern. Some of the species isolated from different bakery products are classified as toxigenic. Visconti and Basilico (1983) for example, identified the ochratoxin-forming *Aspergillus ochraceus* in mouldy bread in Italy. Nevertheless, the natural occurrence of mycotoxins on bread has not been fully demonstrated.

1.3.3 Preservation

There are different ways to increase bread mould-free shelf:

(i) Reducing any mould spores contaminating the bread; essentially by improving bakery hygiene to prevent moulds gaining access. It is also possible to obtain a larger mould-free shelf-life products by packaging before baking, immediately after baking (before the surface temperature falls below 80°C) or cooling, slicing or finishing and wrapping under sterile conditions (Seiler, 1984).

(ii) Destroying or damaging the mould spores which gain access to the surface during the cooling and wrapping processes. These processes can be achieved using UV light, IR or microwave irradiation. This processes have been used for sourdough bread in continental Europe (Seiler, 1984), but although effective, their bad publicity and the increasing demand for mid-processed and "fresh" products from the consumer limits their use. Furthermore, UV irradiation for example, does not penetrate the product so that mould spores inside the loaf will probably not be affected (Legan, 1993).

(iii) Limiting the rate of mould growth. Several strategies can be followed

- reformulating recipes, e.g., in a way to reduce the water availability but without adversely affecting the eating quality of the product or causing changes in volume, shape and texture.
- using novel ingredients like raisin and prune juice concentrate that inhibit fungal growth (Sanders, 1991)
- using chemical preservatives
- using modified atmospheres (MAP) or other packaging techniques (Weng *at al.*, 1999, Abellana *at al.*, 2000). MAP techniques are based on the fact that moulds are oxygen dependent and highly sensitive to carbon dioxide (Portier *et al.*, 1989; Farber, 1991). Thus, achieving environments with low O_2 and high CO_2 contents will protect the wrapped food against aerobic spoilage microorganisms. Further studies have been focused on the development of polymers that contain the

preservative as an active packaging material. In this case, the diffusivity of the preservative from the packaging to the food becomes very important (Han and Floros, 1997)

At the moment mould spoilage of bread and wheat flour-based products is generally prevented by the addition of food grade preservatives such as propionic, sorbic and acetic acids and certain of their salts. The status and use of these materials is controlled in many countries by legislation which limits the type and concentration of preservatives which may be used. This can be affected by the bread type or by other factors such as whether or not the bread is wrapped.

In England and Wales, for example, sorbates are not permitted in bread but it is permitted for flour confectionery goods at levels up to 1000 ppm (Seiler, 1984). In other countries like Germany, Italy or Netherlands, sorbic acid and its salts are authorised in certain types of bread. In the UK, as in many other countries, propionates are the chemical antimicrobials generally used to control mould and bacterial (*Bacillus* spp) spoilage of bread (Legan, 1993). Propionates are used mainly as its sodium, potassium or calcium salts because, although more expensive, they are less corrosive and easier to handle than the liquid acid. Its use is permissible at levels of not more than 0.3% (w/w) of propionic acid equivalent (Anon, 1984). Furthermore, propionates have little or no effect against yeast (Seuer, 1977) which make them highly suitable to control mould spoilage in yeast-raised bread.

But the use of weak acids in bread also has disadvantages. The low absolute efficacy of the propionates means that relatively large high concentrations are needed in order to keep bread and other baked goods free of moulds for more than a few days (Lück and Jager, 1997). At such concentrations serious losses in volume and an adverse effect on odour and flavour occur. Using 0.2% of calcium propionate, for example, a reduction of 5-10% of loaf volume occurs in commercial-scale baking because it reduces the yeast activity and alters the dough rheology. Sorbates have even greater adverse effects (Legan, 1993).

1.3.4 Future

Since the 1980's the bread industry has been working to reduce the number of additives and so called synthetic preservatives in a genuine effort to make bread as natural and fresh as possible (Casdagli, 2000).

As modern consumers prefer minimally processed high-quality foods with few or no additives, several studies have been focused in the last few years on the use of natural preservatives such as plant essential oils. Novel biotechnology techniques have continuously being developed.

Certain combinations of preservatives, e.g. carbon dioxide (in storage and packaging) and ethanol or sorbic acid (Vora *et al.*, 1987; Smith *et al.*, 1988), or vinegar and calcium propionate (0.10% each) (McNaughton *et al.*, 1998), have been shown to be useful measures to improve the life of baked goods.

Finally, Javanainen and co-workers (1993, 1994) have been studying the possibility of producing propionates *in situ* as preservatives through balanced and controlled mixed-culture fermentation in order to retard mould growth and thus to extend the shelf life of the product (Javanainen and Linko, 1993; Javanainen and Linko, 1994; Linko *et al.*, 1997).

Nevertheless, the acceptance in the market of the new long-life 7-day foil loaf, highly treated with preservation techniques, since its launch in 1997 (Casdagli, 2000) suggest that the consumer is not as concerned about natural products as it is for the convenience of a longer shelf-life product.

Chapter 2

AIMS AND OBJECTIVES

2.1 AIMS OF THE PROJECT

Currently, growth of spoilage fungi in intermediate moisture bakery products, specifically bread and cakes, is prevented by the addition of food grade preservatives such as potassium sorbate, calcium propionate or sodium benzoate. However, pressure exists from both consumer and legislation to reduce the amount of chemical preservatives added to foodstuffs (EC Council Directive 95/2/EC). Reduction in the actual permitted and used doses can result in stimulation of growth and mycotoxin production of spoilage yeast and filamentous fungi, which can significantly reduce product shelf-life, influence microbiological safety and thus have important implications for both processing industries and consumers.

The first set of experiments of this project aimed to study the effect of sub-optimal concentrations of existing preservatives (log reduction of the maximum permitted level) on mould growth and ochratoxin A production on wheat flour-based substrates. A representative group of fungal species commonly found in spoiling bakery products was identified. A 2% wheat flour agar and bread analogues were used under different environmental conditions, in order to simulate the intrinsic characteristics of different bakery products currently preserved by salts of organic acids.

Further experiments were focused on the screening and study of new/natural alternative antifungal compounds for bread preservation. Plant essential oils and antioxidants were considered for this purpose.

The final part of the project was aimed at evaluating the effect of different preservation factors (environment and preservatives) on fungal ecophysiology, and to study the possible mechanism of action of both existing and alternative preservatives.

This work was carried out as part of the EU-sponsored project "Natural antifungal systems for prevention of mould spoilage in bakery products" (FAIR PL98-4075)

2.2 OVERALL OBJECTIVES

The main objectives of the project were:

1 To study the effect of sub-optimal concentrations of existing food-grade preservatives on mould growth and toxin production in a range of wheat-based products at different environmental conditions.

2 To screen for essential oils and antioxidants for control mould spoilage in bread products

3 To study the dose-response effect of the most promising alternative antifungal compounds on mould growth under different environmental conditions.

4 To study the effect of environment and sub-optimal concentrations of existing preservatives on species interactions.

5 To study the effect of different type of preservative on hydrolytic enzyme activity in order to compare possible mechanisms of action between existing and alternative preservatives.

To achieved these objectives the following work was carried out:

(i) Effect of sub-optimal concentrations of potassium sorbate, calcium propionate and sodium benzoate on *in vitro* growth of spoilage fungi on wheat flour agar at different environmental conditions (water activity x pH x temperature combinations)

(ii) Effect of sub-optimal concentrations of potassium sorbate and calcium propionate on *in situ* mould growth on bread analogues at 25°C and different water activity and pH levels.

(iii) Screening of essential oils and antioxidants with *in vitro* antifungal activity at a combination of environmental conditions that allows for fast mould growth.

(iv) Effect of dose and environment on *in vitro* and *in situ* antifungal activity of best essential oils and antioxidants

(v) Effect of existing and alternative preservatives on ochratoxin A production by *Aspergillus ochraceus* and *Penicillium verrucosum* on wheat flour agar and bread analogues at different environmental factors.

(vi) Effect of environment and sub-optimal concentrations of potassium sorbate on niche size and niche overlap index (NOI) of bread spoilage fungi.

(vii) Impact of preservatives and environment on hydrolytic enzyme production by a range of spoilage fungi

Figure 2.1 depicts diagrammatically the work carried out in this project.

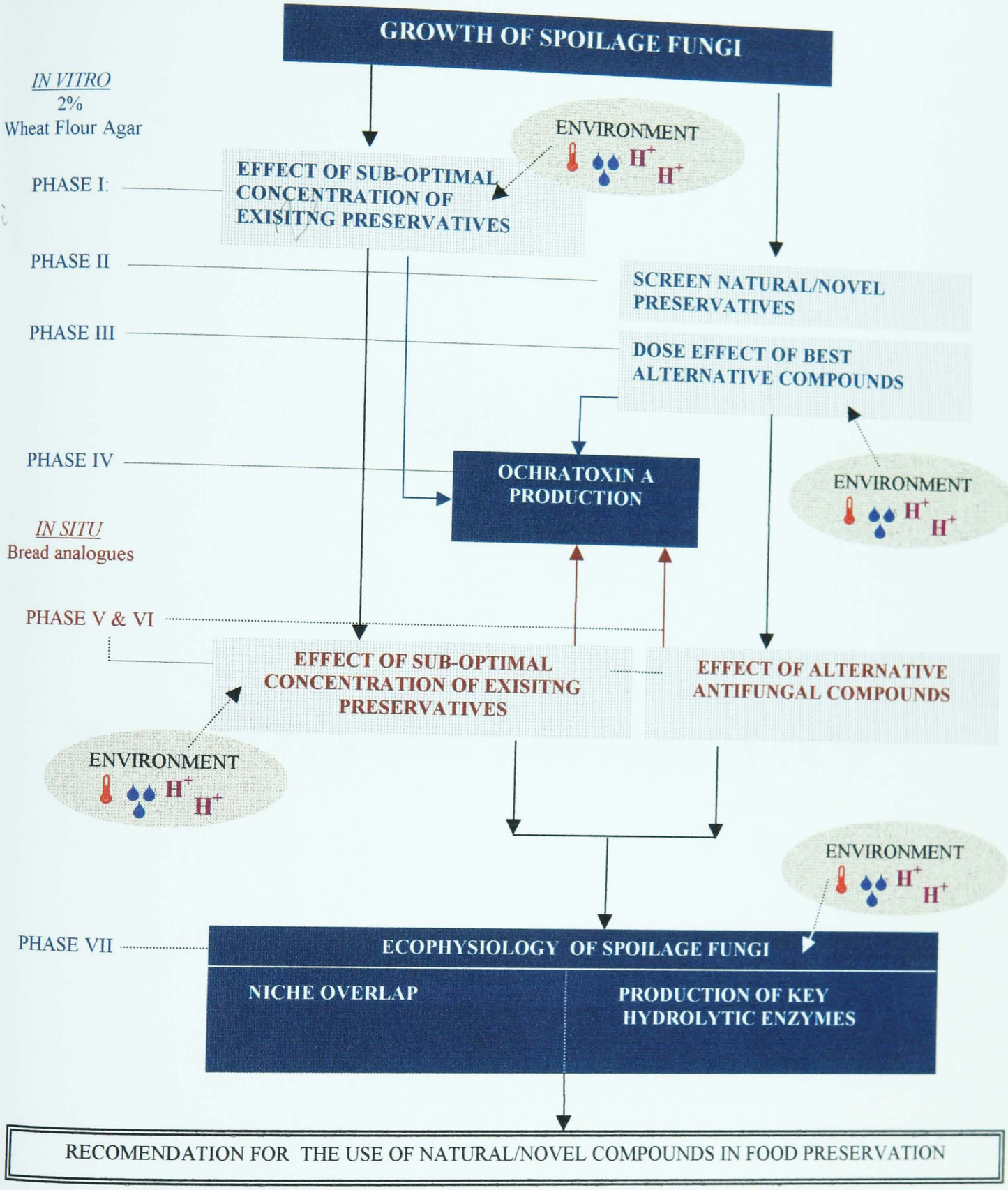


Figure 2.1 Flow chart of project work

Chapter 3

MATERIALS AND METHODOLOGY

3.1 FUNGAL SPECIES AND CULTURE MAINTENANCE

The fungal species used in this study, together with the origin and code of the culture collection are shown in Table 3.1. Plate 3.1 shows the morphology of the different species on MEA after 14 days incubation at 25°C.

During the duration of the project, all species were periodically sub-cultured on MEA (every 2-3 months) and on WFA (weekly, in order to have a constant supply of fresh cultures). *Eurotium* species were only grown on MEA supplemented with 20% sucrose (Merck) to promote its sporulation (ascospores) which was otherwise poor. Spore suspensions of the initial culture on MEA (working culture) were kept at –80°C in a 15% glycerol-water solution.

Using a sterile loop, fungal spores from a MEA working culture were collected and placed in a glass Universal bottle containing 10ml distilled water + 0.01% tween 80 (Merck). The bottles were shaken vigorously for 30 seconds. Petri plates (90 mm diameter) of MEA/WFA were then inoculated with the spore suspension by spread plating to obtain an even covering on the surface of the agar (fresh culture). The plates were placed in polyethylene bags and incubated for 7-10 days at 25°C.

3.2 SUBSTRATES FOR FUNGAL GROWTH

For all experiments, batches of wheat flour were kindly provided by Campden and Chorleywood Food Research Association Ltd. (CCFRA), Chipping Campden, UK.

3.2.1 Basic Nutritional Media

(i) Wheat Flour Agar (WFA): 2% wheat flour agar was prepared by homogenising 2% (w/v) of wheat flour and 2% (w/v) of technical agar No 3 (Lab M) in distilled water. Once well homogenised, the substrate was autoclaved for 15 minutes at 121°C and 1 atm overpressure, and poured when the temperature was approximately 50°C, into standard 90 mm sterile Petri plates (20ml media/plate). WFA plates were kept in sealed polyethylene bags at 4°C until inoculation for a maximum period of 14 days.

Table 3.1 Fungal species used in this study together with their origin and culture collection code.

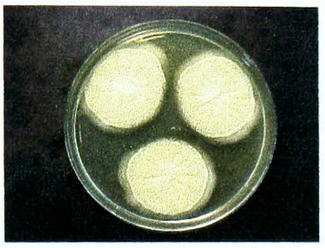
SPECIES	*ORIGIN*	*CODE*
Aspergillus ochraceus Wilhelm	Wheat (Sweden[1])	SLV505/CBS290.95
Penicillium corylophilum Dierckx	Rye Bread (Denmark[2])	IBT6978
Penicillium verrucosum Dierckx		
strain M450	Wheat (Sweden)	M450
strain M453	Wheat (Sweden)	M453 (SLV493/CBS815.96)
strain PV3	Wheat (Sweden)	12996
Eurotium repens de Bari	Almond cake (Denmark)	IBT18000
Penicillium roquefortii Thom	Cheese (Denmark)	IBT18687
Cladosporium herbarum (Pers.) Link	Wheat (UK[3])	CH1

1 National Food Administration, Uppsala, Sweeden
2 Technical University of Denmark, Lyngby, Denmark
3 Applied Mycology Group, Cranfield University, UK

Penicillium corylophilum

Penicillium roquefortii

Aspergillus ochraceus

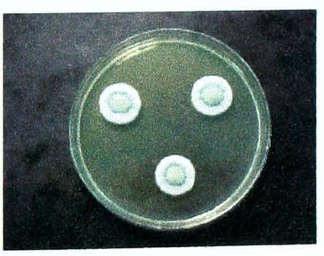


Cladosporium herbarum

Eurotium repens

Penicillium verrucosum PV3

Penicillium verrucosum M450



Penicillium verrucosum M453

Plate 3.1 Colonies of *Aspergillus ochraceus, Eurotium repens, Cladosporium herbarum, Penicillium corylophilum, Penicillium roquefortii* and strains M450, M453 and PV3 of *Penicillium verrucosum* on MEA after 14 days incubation at 25°C.

To study the effect of environmental conditions on fungal ecophysiology and growth, WFA was modified, prior to autoclaving, to different a_w and pH levels. The a_w of the substrate was modified with glycerol (GPR, Merck) and different pH values were achieved with citric acid 0.1M (AnalaR, Merck) - sodium phosphate 0.2M (AnalaR, Merck) buffer (Mc Ilvaine's buffer; Dawson *et al.*, 1987). The relationship between the amount of added glycerol and a_w of the final media was determined experimentally (Figure 3.1).

(ii) Malt Extract Agar (MEA): Commercial MEA (Merck) was supplemented with 1% technical agar No 3. Once the components were homogenised, the substrate was autoclaved and poured as mentioned above and following the supplier recommendations. Prior to inoculation, all prepared media was stored at 4°C for a maximum period of time of 14 days.

3.2.2 Preparation of Bread Analogues

Recipes for bread analogue at different a_w and pH levels were developed. The a_w was modified from its intrinsic value (0.98-0.99) with the addition of different amounts of glycerol. The relationship between grams of glycerol versus a_w values was experimentally obtained as shown in Figure 3.2.

For 100g of flour, 35ml of 0.1M of citric acid and 10ml of 0.1M sodium hydroxide (Sigma) solutions were added to achieve pH levels of 4.5 and 6 respectively. Bread analogues were made by mixing 100g flour, 5g butter, 0.5g salt, 0.5g sugar and 0.5g yeast (Patterson & Damoglou, 1986) with the adequate amounts of glycerol, buffer and water for a total volume of 70 ml.

Two different procedures for bread analogue preparation were compared: slicing before and after autoclaving. Shape, a_w and pH uniformity between slices together with the period of time for which the analogue remained sterile were the parameters used for comparison. Results are shown in Table 3.2. Based on these results and because of the much better sterility conditions achieved, it was decided to slice the bread dough before autoclaving.

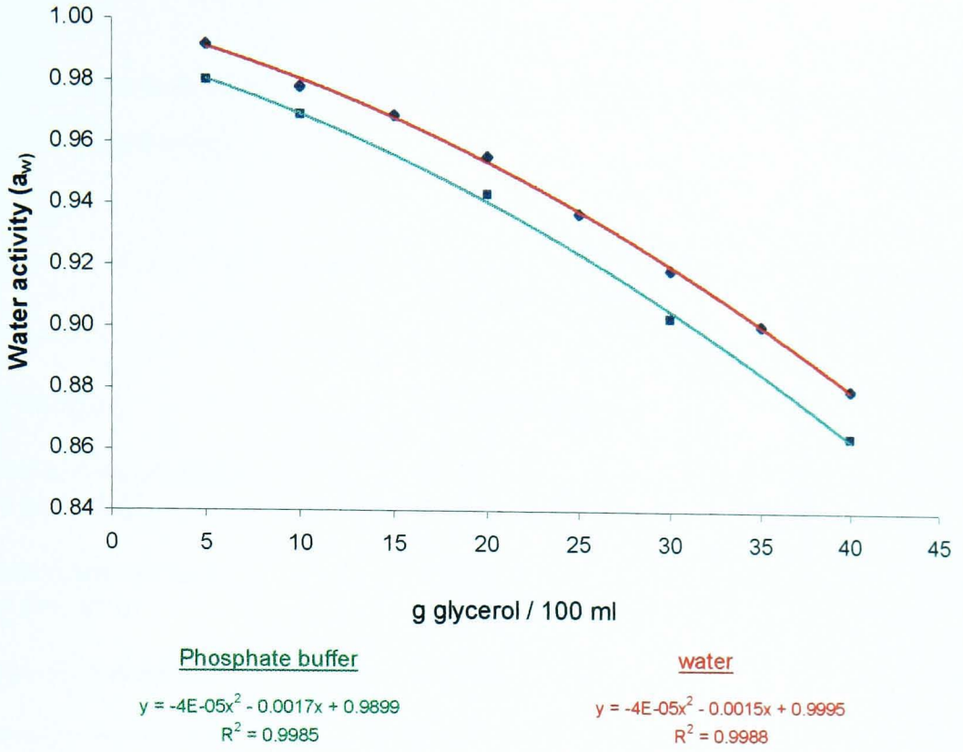


Figure 3.1 Calibration curve of grams of glycerol versus a_w level for a 2% WFA prepared with (a) water and (b) with phosphate buffer solution. Points are means of three replicates (standard deviations <0.01)

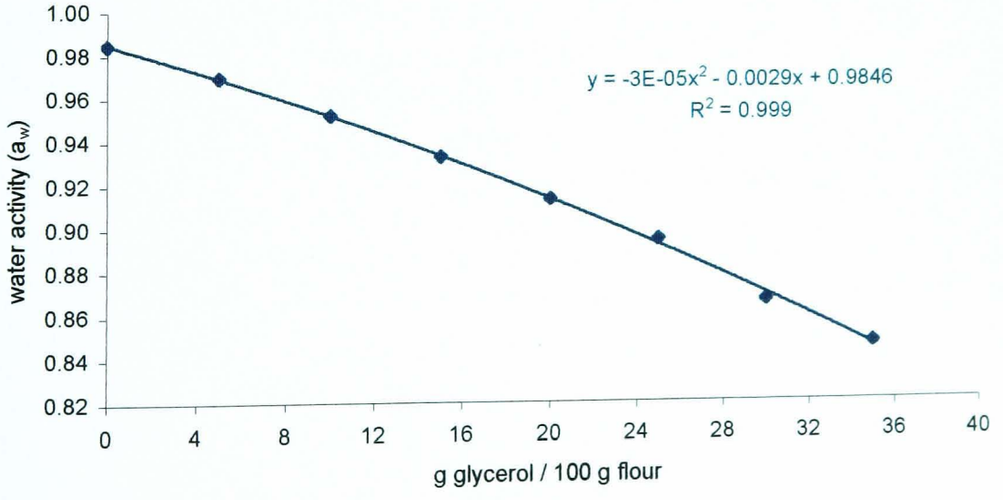


Figure 3.2 Calibration curve of grams of glycerol versus a_w level for bread analogue. Points are means of thirty replicates from three different experiments (standard deviations < 0.01).

Table 3.2 Comparison of two different protocols for the bread analogue preparation (values are means of three replicates).

	Procedure	
	Sliced before autoclave	*Sliced after autoclave*
Period of sterility	up to 21-30 days	7-15days
pH uniformity between slices (standard deviation)	0.077	0.099
a_w uniformity between slices (standard deviation)	0.0054	0.078
Shape uniformity achieved	uniform	variable

Samples of 15g of the bread dough were shaped into approximately 60 mm diameter discs and placed between two squares of aluminium foil (15cm^2 approx). Packs of 20 bread slices were sealed in aluminium foil and autoclaved at 121°C and 1 atm for 15 minutes. When still hot, the slices were sprayed with isopropanol (Fisher) and placed into sterile 90 mm Petri plates.

Before inoculation, plates with bread discs were kept 24h in sealed polyethylene containers containing beakers of glycerol-water solutions (Dallyn and Fox, 1980) in order to accurately maintain equilibrium relative humidity (e.r.h.) of treatments. Under these conditions adequate a_w levels of bread discs were maintained over the experimental period of time (30 days) with maximum a_w change of 0.02.

3.2.3 Control of a_w and pH levels

Water activity of all prepared media (WFA and bread analogues) was systematically measured with an AQUA LAB a_w meter. The a_w meter was calibrated prior to use against saturated salts solutions with known a_w (0.750, 0.973 a_w and 1.000 a_w). Two plates of WFA/bread were used and the mean of three consecutive a_w measures recorded.

pH levels of WFA plates and bread analogues were systematically checked with a pH/mV meter (Hanna HI 8424) supplied with a gel filled electrode for pH measures of solid substrates, and a temperature probe. The meter was also calibrated daily before its use in the range of pH from 4.01 to 7.01, and from 7.01 to 10.01 when required.

3.3 SUBSTRATE INOCULATION AND INCUBATION

A flow chart of the culture protocol followed throughout the project is shown in Figure 3.3. Using a sterile loop, fungal spores from fresh cultures on WFA (see figure 3.3) were collected into sterile Universal bottles containing 10ml distilled water + 0.01% tween 80 (Merck). Spore concentration was measured with a haemocytometer (Weber) and adjusted to approximately 10^6 spores ml^{-1}. Spores were counted using an optical microscope (Olympus, B201) at 40x magnification.

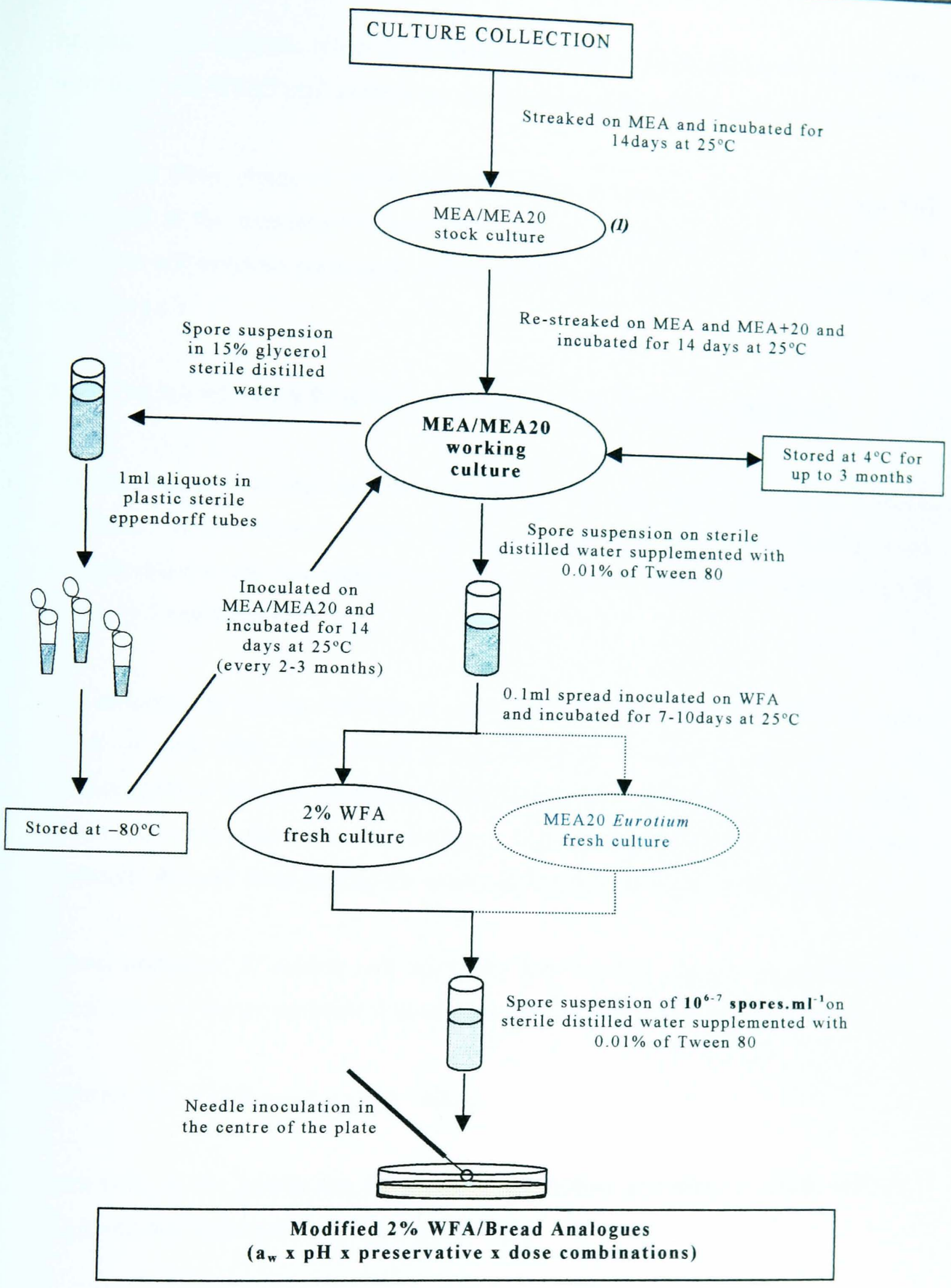


Figure 3.3 Fungal culture maintenance and inoculation protocol flowchart.

(1) MEA supplemented with 20% sucrose (MEA20) used only for cultures of *Eurotium* spp.

At least, three replicate plates per treatment for both *in vitro* and *in situ* experiments were used. All WFA/bread plates were needle-inoculated centrally using a sterile loop.

Inoculated Petri plates of WFA were enclosed in sealed polyethylene bags and incubated at the experimental temperature. Plates containing bread analogues were placed in polyethylene containers with glycerol-water solutions in order to maintain a constant e.r.h.

3.4 PARAMETERS FOR MEASUREMENT OF FUNGAL GROWTH

Two parameters were used as a measure of mould growth on 2% WFA and on bread analogues: Length of the lag phase prior to growth and the growth rate. The *lag phase* was calculated as the time required for the colony to grow beyond the inoculation zone (typically 5 mm diameter).

Two measures of colony diameter of each replicate plate, in two directions at right angles to each other, were taken over a period of 30 days. A minimum of 7-8 measurements of temporal mycelial extension were used for each plot to obtain accurate growth rate values. *Growth rates* (mm day^{-1}) were calculated as the slope of the linear regression obtained from plotting the colony radius of the replicates against time.

A third parameter, *% growth rate inhibition*, was used to estimate the antimicrobial effectiveness of the preservative studied. This parameter was calculated as follows:

$$\textit{Inhibition}\ (\%) = (GR_{NP}-GR_p) \times 100 / GR_{NP}$$

where GR_{NP} is the growth rate on control plates without preservative added, and GR_p the growth rate in the presence of the preservative.

3.5 EFFECT OF EXISTING WEAK-ACID PRESERVATIVES ON GROWTH OF SPOILAGE FUNGI UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

3.5.1 *In vitro* studies on 2% wheat flour agar

An initial study on the effect of sub-optimal concentrations of existing food grade preservatives potassium sorbate, calcium propionate and sodium benzoate (Aldrich) was carried out over a wide range of a_w and pH simulating a variety of bakery products which are commonly preserved with these organic acids.

WFA was modified to 4 different a_w levels, 0.95, 0.90, 0.85 and 0.80, and three pH levels, 4.5, 6 and 7.5. Preservatives were added to the substrate at doses of 3000 ppm (actual maximum permitted level) and at sub-optimal concentrations of 300 and 30 ppm (w/w). The required amount of preservative was homogenised with the medium before autoclaving. Plates of modified WFA were inoculated with the appropriate spore suspension (see section 3.3) and incubated at two temperatures, 15 and 25°C.

3.5.2 *In situ* studies on bread analogues

After analysis of the results obtained in the *in vitro* experiments, parameters for the studies on bread analogues were set up. Bread analogues were modified to a narrower range of a_w (0.97, 0.95 and 0.93) and pH (4.5 and 6) in order to simulate the intrinsic characteristics of white bread which is characterised by high moisture contents and low acidity.

Potassium sorbate and calcium propionate were the organic acids used *in situ* at the sub-optimal concentration of 300 ppm (w/flour w) and the maximum of 3000 ppm (w/flour w). Preservatives were incorporated into the bread analogues as an additional ingredient prior to autoclaving. A total of 18 different a_w x pH x dose combinations per preservative type were studied at 25°C.

3.6 EFFECT OF ANTIOXIDANTS AND ESSENTIAL OILS ON GROWTH OF SPOILAGE FUNGI UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

3.6.1 In vitro studies on 2% wheat flour agar

(ii) Screen for alternative natural/novel compounds for mould growth control in bread

In vitro antifungal activity of four anti-oxidants (Sigma): butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *n*-propyl gallate, and propyl paraben, and 20 plant essential oils was screened against *Aspergillus ochraceus, Penicillium corylophilum, Penicillium verrucosum* (strains M450, M453 and PV3) and *Cladosporium herbarum.* The study was initially carried out at a single combination of environmental conditions represented by 0.95 a_w, pH 6 and 25 °C.

Anti-oxidants and essential oils were diluted in methanol (1% w/media w) (Fisher, analytical grade) and incorporated and mixed with the substrate after the sterilisation process when the temperature of the substrate was about 50°C. Plates with the same amount of methanol only were used as positive controls. Phenolic anti-oxidants were added to the substrate at three different doses, 100, 500 and 1000 ppm (w/w), and essential oils at only one dose, 500 ppm (w/w). All essential oils were kindly given by F.D.Copeland & Sons, Ltd. (London). Table 3.3 shows the list of essential oils tested together with their commercial code.

(ii) Effect of dose of best antioxidants / essential oils on *in vitro* control of mould growth at different environmental conditions

For the best alternative compounds, a more detailed screen was carried out. Studies were focused on their possible application in bread preservation. Water availabilities of 0.97, 0.95 and 0.93 a_w and pH levels of 4.5 and 6 were tested. Anti-oxidants and essential oils were used in increasing concentrations from 0 to 50, 100, 150, 200, 500 and 1000ppm (w/w) and added to WFA as previously detailed. Media with methanol but no antifungal compound were again used as a control.

Table 3.3 Essential oils screened for antifungal activity

ESSENTIAL OIL	*COMERCIAL CODE **	*ESSENTIAL OIL*	*COMERCIAL CODE*
BASIL- *Lynalool type*	H.82T	MARJORAM- *Spanish*	H.95T
BASIL- *Methyl Chavicol type*	H.83T	NUTMEG	H.97T
BAY	H.85T	ORANGE- *Brazilian*	H.98T
CINNAMON LEAF	H.84T	PEPPERMINT-*Yakima*	H.99T
CLOVE	H.86T	PINE-*Sylvestris*	H.166T
EUCALYPTUS- *Chinese*	H.87T	ROSEMARY-*Spanish*	H.101T
GINGER- *Indian*	H.91T	SAGE-*Spanish*	H.102T
GRAPEFRUIT- *Israeli*	H.92T	SPEARMINT-*American*	H.103T
LEMONGRASS	C.167T	SWEET FENNEL	H.88T
LIMES – *Mexican*	H.94T	THYME-*Spanish*	H.104T

* F.D. Copeland & Sons, Ltd. (London)

3.6.2 *In situ* studies on bread analogues

An initial study on the suitability of anti-oxidants and essential oils on *in situ* control of mould growth was carried out at 0.97 a_w, pH 6 and 25°C. Concentrations of 100, 300, 500 and 1000 ppm (w/flour w) were tested. Compounds were added to the bread analogue as methanol solutions prior to autoclaving.

3.7 EFFECT OF PRESERVATIVES AND ENVIRONMENTAL CONDITIONS ON OCHRATOXIN A PRODUCTION BY *A.OCHRACEUS* AND *P.VERRUCOSUM*

Ochratoxin A (OTA) production by the ochratoxigenic species *Aspergillus ochraceus* and *Penicillium verrucosum* (strains PV3, M450 and M453) was investigated in both *in vitro* and *in situ* studies at different environmental conditions and in the presence of different types and concentrations of existing and novel/natural preservatives.

3.7.1 Sampling

(i) In vitro studies

Initially, a temporal study on the potential of *A.ochraceus* and *P.verrucosum* for OTA production on 2% WFA was carried out at pH 6, 25°C and three different a_w levels, 0.97, 0.95 and 0.93. Effect of sub-optimal concentrations of potassium sorbate and comparison between existing and natural/novel preservatives over time were subsequently studied.

Potassium sorbate was used at sub-optimal concentrations of 150 and 300 ppm (w/w) and 150ppm of BHA, PP and essential oils of thyme, clove, cinnamon and bay were used for comparison. A total of 18 replicates per treatment and species were inoculated as described in section 3.3. After 7, 14, 21, 28, 35 and 54 days of incubation, three replicates per treatment and species/strain were processed for OTA quantification.

(ii) In situ studies

As with *in vitro* studies, an initial temporal study on the potential for OTA production by *A.ochraceus* and *P.verrucosum* on bread analogues was carried out at three a_w (0.97, 0.95 and 0.93) and two pH levels (4.5 and 6) at 25°C. After 7,14, 21, 28 and 35 days three replicates per species/strain and treatment were analysed.

Subsequently, the effect of dose and type of preservatives on these fungi were studied at the time when maximum production of OTA in the temporal study was found (28 days). Two concentrations (300 and 3000 ppm) of existing preservatives potassium sorbate and calcium propionate were assayed. The anti-oxidants BHA and PP and essential oils of thyme, clove, cinnamon and bay were screened at 1000 ppm concentration only. Due to the variability in OTA production, controls with no added preservative, were made and analysed again for every a_w-pH combination studied.

3.7.2 Toxin extraction

(i) In vitro studies

Ochratoxin A extraction was carried out with methanol (Abarca *et al.*, 1994). A recovery assay using this technique on WFA was conducted with a mean recovery of 87.7% (standard deviation 6.8, Table 3.4).

Each agar plate (6-10g agar) was cut and placed into 250ml conical and 50ml of methanol (Fisher, HPLC grade) added. Flasks were sealed with parafilm (Gallenkamp) and shaken on an orbital shaker (IKA HS 501 Digital) for one hour at room temperature. Aliquots of the methanol extract were placed in a 1.5ml plastic Eppendorff tubes and centrifuged in a bench top microfuge (Beckman Lite) at 13000 rpm for 10 minutes. Supernatants were filtered through a 0.2μm syringe filters (Whatman) into 1ml amber HPLC vials (Fisher) and stored at -20°C until processed.

Table 3.4 Percentage of ochratoxin A (OTA) recovery from 2% wheat flour agar and bread analogues extracted with methanol and 1 hour shaking period (values are means of three replicates).

OTA added to media (μg)	**OTA RECOVERED (%)**	
	From WFA	
	mean	*std deviation*
5	81.6	2.3
10	93.7	7.6
20	88.2	2.7
	From Bread Analogues	
	mean	*std deviation*
5	50.6	4.8
10	51.1	5.0
20	63.3	2.0

(ii) In situ studies

Some modifications to the extraction protocol used for *in vitro* studies were needed because (a) the percentage of recovery of OTA from bread analogues with the initial methodology was less than 55% (see Table 3.4) and (b) mould colonies on bread analogues were much more stabilised and pigmented than on WFA resulting in imprecise chromatograms.

To increase the percentage recovery it was decided to extend the shaking time to 10-12 hours. Bread discs (10-15g) were cut and placed into 250ml conical flasks containing 50ml methanol. Flasks were shaken overnight on an orbital shaker at room temperature. This modification led to a higher percentage of extraction and recoveries of 75.4% (standard deviation 9.2% ,Table 3.5).

The second modification of the extraction protocol involved the clean up process. With a new methodology, toxin extracts were filtered through filter paper in plastic funnels containing 5-10g of the filter agent Celite (BDH). To ensure that the toxin did not bind with Celite, a methanol solution of pure ochratoxin A was also filtered following the same procedure. No toxin retention was observed.

When necessary, extracts obtained were filtered again through a 0.2μm syringe filter. With this modification, HPLC chromatograms were significantly improved and toxin peaks clearly detected (Figure 3.4). Final filtrates were transferred into 1ml HPLC vials (Fisher) and kept at -20°C until samples were successfully processed.

Table 3.5 Percentage of ochratoxin A (OTA) recovery from bread analogues extracted with methanol and a 12 hour shaking period (values are means of three replicates).

OTA added to media (μg)	**OTA RECOVERED (%)**	
	From Bread Analogues	
	mean	*std deviation*
5	72.2	5.1
10	73.8	13.0
20	80.5	9.4

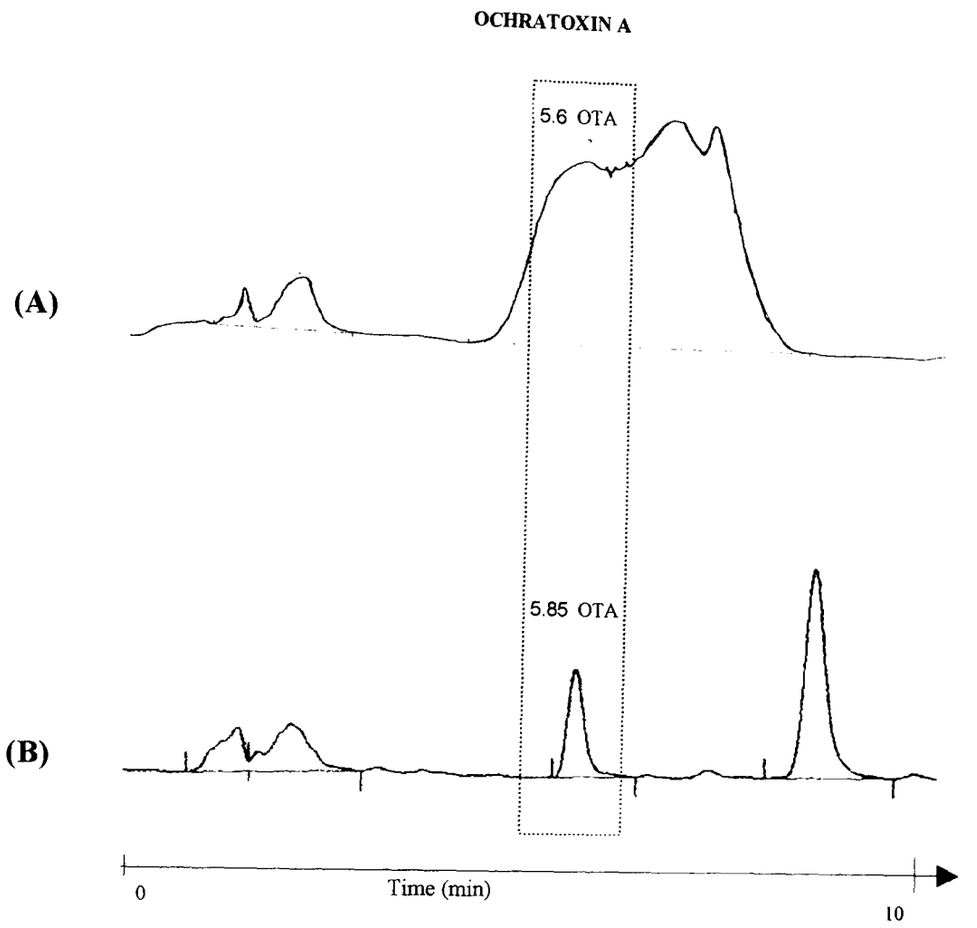


Figure 3.4 Chromatograms of extractions of ochratoxin A (OTA) carried out (A) without celite and (B) with celite, from a culture of *Penicillium verrucosum* M450 on bread analogue at 0.97a_w, pH 6 and in the presence of 300 ppm of calcium propionate after 28 days of incubation.

3.7.3 Quantification

OTA contents were measured using High Performance Liquid Chromatography (HPLC). An HPLC (Waters 600E System controller) with a fluorescence detector (Waters 470) and a auto-sampler (Waters 712 WISP) were used. Conditions for OTA detection were as follows.

Mobile phase	Acetonitrile (57%) : Water (41%) : Acetic acid (2%) (Fisher, HPLC grade)
Column	5µl C18 (150 x 4.6 mm) (Phenomenex Luna)
Pre column	Security Guard provided with 4 x 3 mm cartridges (Phenomenex Luna)
Excitation λ	330 nm
Emission λ	460 nm
Flux	1 $ml.min^{-1}$
Volume of injected sample	50µl
Toxin standards	150, 300, 600 and 1000 $ng.ml^{-1}$ (methanol solutions) (Sigma)
Retention time	6 minutes (see figure 3.4)
Run time	10 minutes for WFA extracts 30 minutes for bread analogues extract

Under these experimental conditions, a fluorescence detection of up to 1-1.2 µg ml^{-1} OTA was possible. Figure 3.5 depicts the chromatogram of a methanol solution of pure ochratoxin A (0.15 µg ml^{-1}). A calibration curve of OTA contents (µg ml^{-1}) versus peak area (mV.min) was made for every sample run. Figure 3.6 shows the calibration curve obtained in one run. Chromatograms and toxin contents were analysed with the aid of the software program Kroma 2000 with chromatogram base line adjustments when necessary.

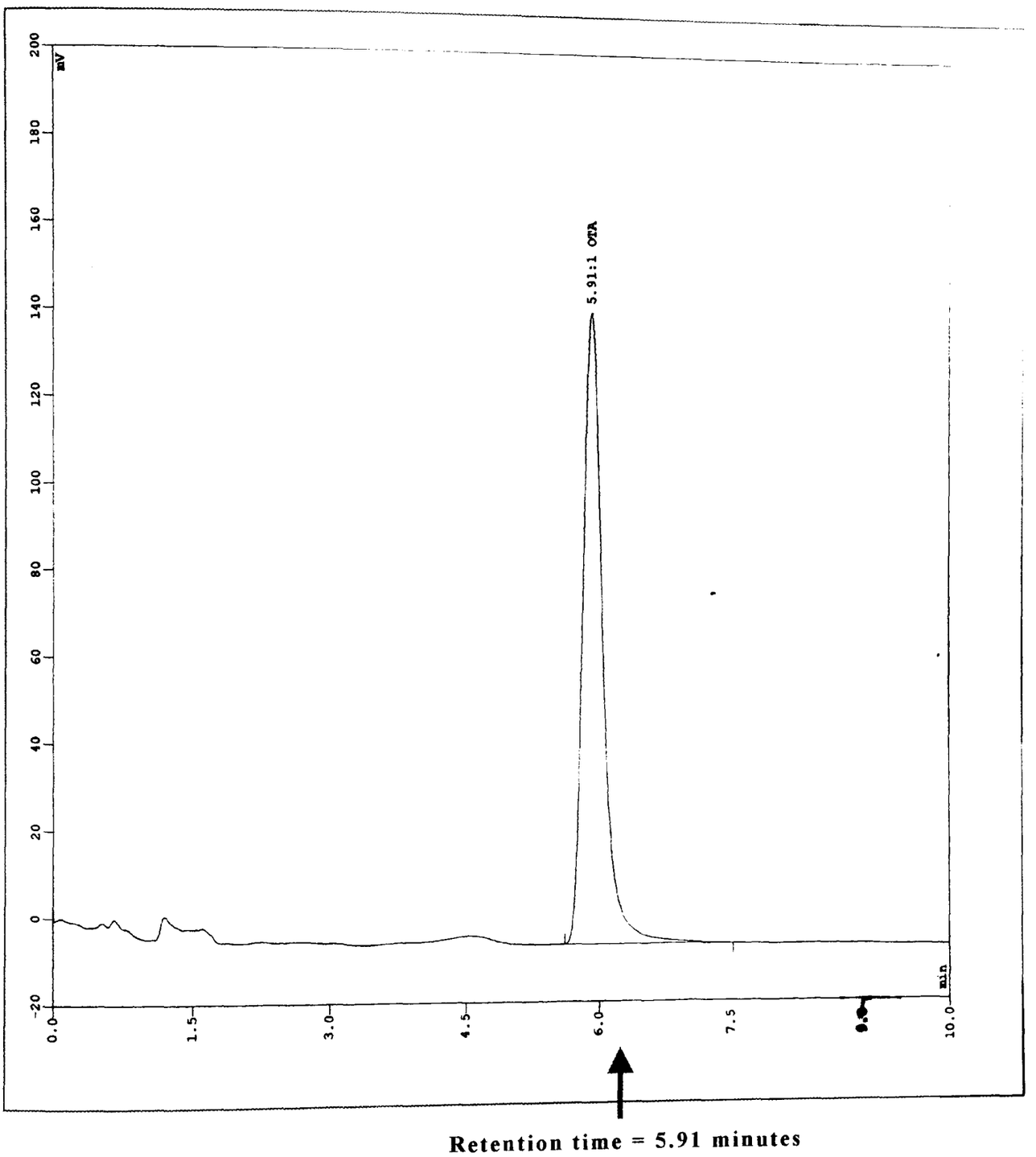


Figure 3.5 Chromatograms of a pure ochratoxin A (OTA) solution in methanol (0.15 μg ml^{-1}).

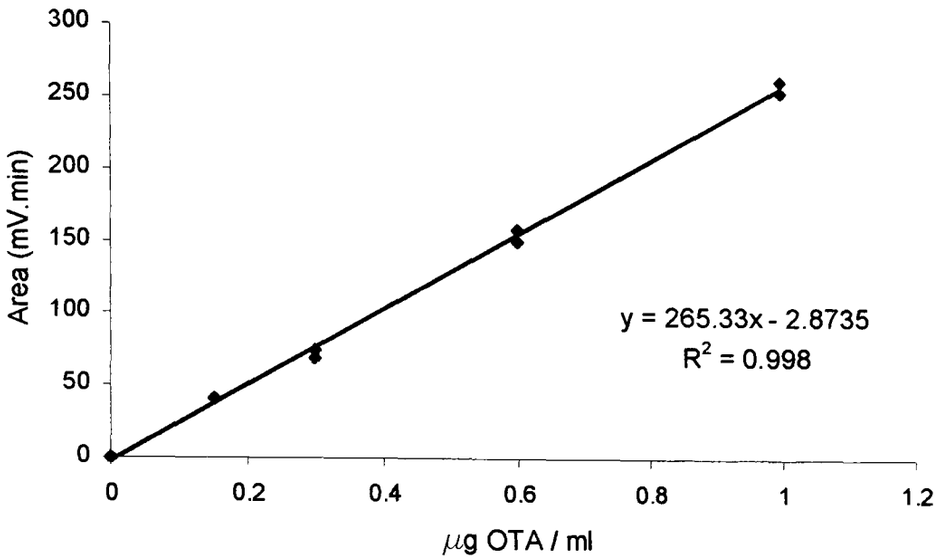


Figure 3.6 Calibration curve of μg ml^{-1} of ochratoxin A versus peak area (mV.min)

3.8 CARBON SOURCE UTILISATION PROFILES AND NICHE OVERLAP BETWEEN SPOILAGE FUNGI IN THE PRESENCE/ABSENCE OF SUB-OPTIMAL CONCENTRATIONS OF POTASSIUM SORBATE

Niche overlap experiments were conducted in order to study the effect of sub-optimal concentrations of weak-acid preservatives on the nutritional competition between species of spoilage moulds on bread products.

Seven fungal species were tested for their ability to catabolize different carbon sources. The substances studied were carbohydrates, amino acids and vitamins (Table 3.6) which constitute some of the main c-sources present in bread (Belitz and Grosch, 1999). Sterile microtitre plates (48 wells, Sterilin) provided with a lid and with a well capacity of 1 ml were used. The carbon source utilisation profiles at different environmental conditions (a_w x pH x T combination) and in the presence or absence of a sub-optimal concentration of potassium sorbate were also studied.

3.8.1 Media preparation

A liquid minimal medium with potassium nitrate (Sigma) as a source of nitrogen was used as the basic substrate (Table 3.7). Carbon compounds were incorporated into the media at a concentration of $9.1x10^{-3}$ g C ml^{-1} (carbon equivalent to 2.28% (w/v) glucose) that represented a final concentration of $8x10^{-3}$ g C ml^{-1} $well^{-1}$ (carbon equivalent to 2% (w/v) glucose).

To study the effect of environmental conditions on carbon catabolism, the minimal medium was modified to different environmental combinations: four a_w (0.97, 0.95, 0.93 and 0.90), two pH (5 and 6.5) and two temperature levels (15 and 25°C). The effects of these conditions were analysed both with and without the addition of 300 ppm of potassium sorbate. The different pH and a_w levels were achieved with phosphate buffer (10mM, Sigma; Dawson *et al.*, 1987) and NaCl (Sigma; Dallyn and Fox, 1980) respectively, since their addition did not represent an additional source of carbon.

Table 3.6 Carbon sources and concentration used in carbon source utilisation profile experiments.

CARBON SOURCE		Formula	*g* C/mol	% compound (w/v) (equivalent to 9.1mgC/ml)
Aminoacids				
L-Cysteine	(SIGMA)	$C_3H_7NO_2S.HCl$	36	3.98
L-Leucine	(SIGMA)	$C_6H_{13}NO_2$	72	1.65
L-Alanine	(SIGMA)	$C_3H_7NO_2$	36	2.25
L-Arginine	(SIGMA)	$C_6H_{14}N_4O_2$	72	2.20
D-L-Threonine	(SIGMA)	$C_4H_9NO_3$	48	2.25
L-Serine	(LANCASTER)	$C_3H_7NO_3$	36	2.68
L-Valine	(LANCASTER)	$C_5H_{11}NO_2$	60	1.78
L-Histidine	(LANCASTER)	$C_6H_9N_3O_2$	72	1.96
L-Methionine	(LANCASTER)	$C_5H_{11}NO_2S$	60	2.26
L-Isoleucine	(ACROS)	$C6H13NO_2$	72	1.66
Glycine	(LANCASTER)	$C_2H_5NO_2$	24	2.85
L-Proline	(SIGMA)	$C_5H_9NO_2$	60	1.74
L-Lysine	(LANCASTER)	$C_6H_{14}N_2O_2$	72	1.85
Carbohydrates				
α-cellulose*	(SIGMA)	-	-	2.28
α-D-Galactose	(BDH)	$C_6H_{12}O_6$	72	2.28
D-Raffinose	(LANCASTER)	$C_{18}H_{32}O_{16}.5H_2O$	216	2.50
D(+)-Xylose	(SIGMA)	$C_5H_{10}O_5$	60	2.28
Starch*	(SIGMA)	-	-	2.28
α-D-Glucose	(SIGMA)	$C_6H_{12}O_6$	72	2.28
Maltose	(SIGMA)	$C_{12}H_{22}O_{11}.H_2O$	144	2.28
D(-)-Fructose	(SIGMA)	$C_6H_{12}O_6$	72	2.28
Sucrose	(SIGMA)	$C_{12}H_{22}O_{11}$	144	2.16
Glycerol	(MERK)	$C_3H_8O_3$	36	2.33
L-Arabinose	(SIGMA)	$C_5H_{10}O_5$	60	2.28
Gluten*	(SIGMA)	-	-	2.28
Vitamins				
Thiamine	(SIGMA)	$C_{12}H_{17}ClN_4OS$	144	2.13
Riboflavine	(SIGMA)	$C_{17}H_{20}N_4O_6$	204	1.68
Nicotinic acid (niacin)	(SIGMA)	$C_6H_5NO_2$	72	1.55
DL-Panthoteic acid	(SIGMA)	$C_9H_{16}NO_5.1/2Ca$	108	2.01
L-Ascorbic acid	(SIGMA)	$C_6H_8O_6$	72	2.22
(+)-α-Tocoferol	(SIGMA)	$C_{29}H_{50}O_2$	348	1.13
Folic acid	(SIGMA)	$C_{19}H_{19}N_7O_6$	228	1.76

*added into the media at a concentration of 2.28% (w/v)

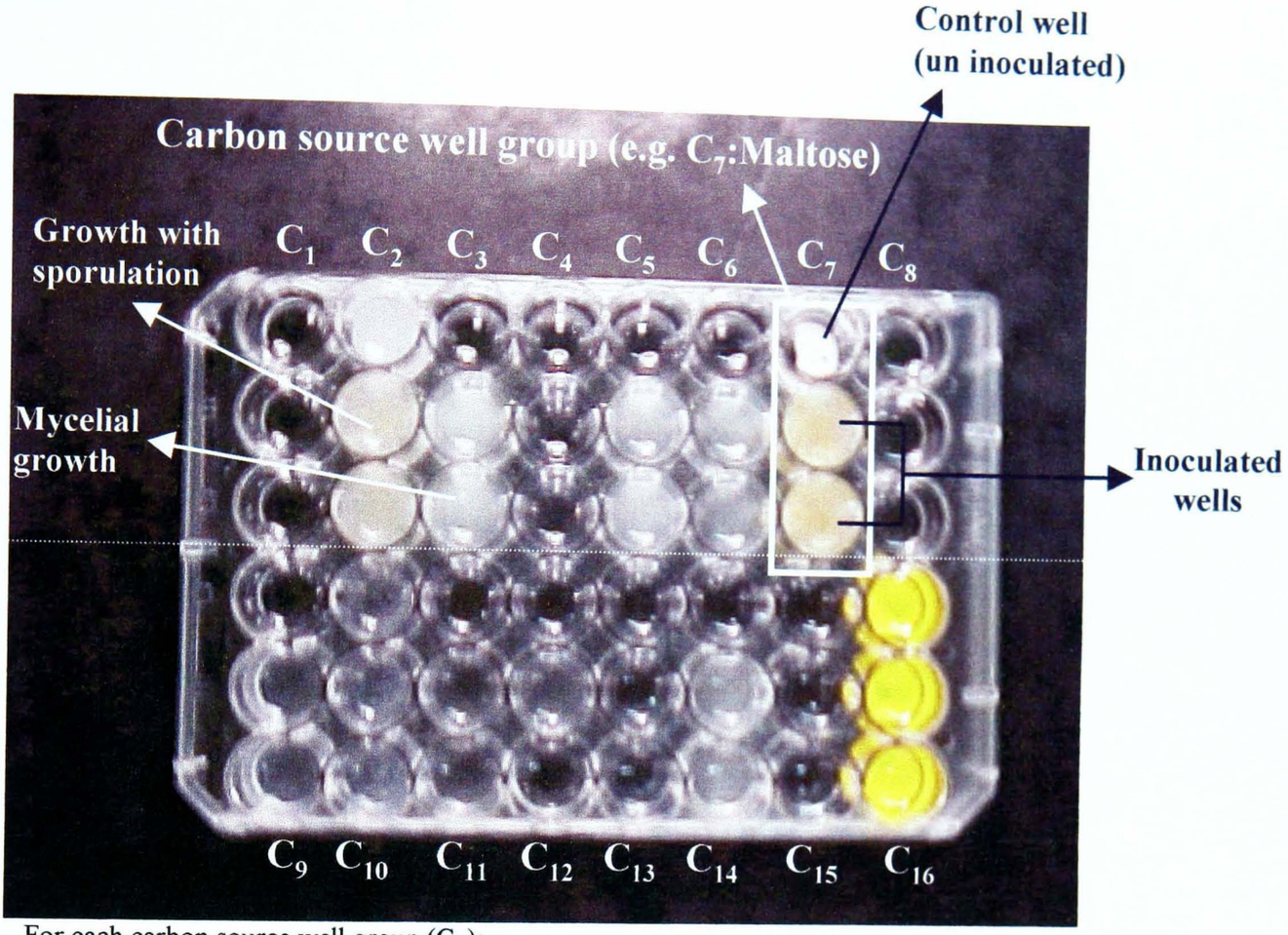
Taking into account that NaCl reduces the pH value of a buffered solution, a pH-g NaCl calibration curve was made (Figure 3.7). The combinations of the pH of buffered solutions and grams of salt necessary for different a_w-pH treatments are show in Table 3.8. Potassium sorbate was incorporated into the minimal medium at a concentration of 0.034 % (w/v) (300 ppm final concentration per well).

Carbon sources were weighed and placed individually in 50ml plastic sterile Universal bottles where 40 ml of the buffer-NaCl solution previously autoclaved and cooled was added under sterile conditions. Aliquots of 700μl of each carbon source solution were placed into three wells of the 48-well sterile microtitre plate. Two plates per species and a_w x pH x T combination were used.

3.8.2 Spore suspension preparation and inoculation

Under sterile conditions, fungal spores of the different species from fresh cultures on 2% WFA were harvested and individually placed into sterile plastic Universal bottles containing 20ml of distilled water. Bottles were shaken vigorously for 10-15 seconds and centrifuged in a bench top microfuge for 15 minutes at 3000 rpm. After discarding the supernatant with care, another 20 ml of sterile water were added. The washing process was repeated three times. After the third wash, spores were resuspended with the adequate buffer-NaCl sterile solution and the spore concentration was adjusted to 10^6 spores ml^{-1}.

For each CS solution well group, 100 μl of the spore suspension was added to two wells and 100 μl of Buffer-NaCl solution was used as a negative control and added to the third well. Microtitre plates were placed in sealed polyethylene bags and incubated at 15 and 25°C. After 14 days incubation and with the aid of a binocular magnifier (Olympus), presence or absence of growth was recorded. Plate 3.2 exemplifies the microtitre plate technique followed.



For each carbon source well group (C_i):

	Carbon source solution	Spore suspension	Buffer-NaCl
Well 1 (control well)	700 µl	-	100 µl
Well 2	700 µl	100 µl	-
Well 3	700 µl	100 µl	-

Plate 3.2 Representation of the microtitre plate technique for the determination of fungal niches. The example illustrates the utilisation profile of *Aspergillus ochraceus* in the presence of 16 different carbon sources at 0.93 a_w, pH 5 and 25°C.

Table 3.7 Minimal medium composition

COMPONENT	CONCENTRATION (%, (w/v))	
	*INITIAL**	*FINAL***
$NaNO_3$	0.23	0.2
$MgSO_4.7H_2O$	0.06	0.05
Glucose *1	2.28	2.00
K_2HPO_4 *2	0.17	0.17
KH_2PO_4 *2	0.13	0.13

* Concentration in the carbon source solution made
** Final concentration in the well
*1 Carbon source of reference
*2 From the phosphate buffer solution (10mM)

Table 3.8 Amounts of NaCl and pH of buffered solution necessary for the different a_w-pH treatments.

TREATMENT			
a_w	*pH*	**BUFFER pH**	**g NaCl/100ml BUFFER** *(Dallyn and Fox, 1980)*
0.97	5	5.8	5.31
	6.5	7.2	5.31
0.95	5	5.9	9.3
	6.5	7.5	9.3
0.93	5	6.2	11.98
	6.5	7.6	11.98
0.90	5	6.3	16.56
	6.5	7.8	16.56

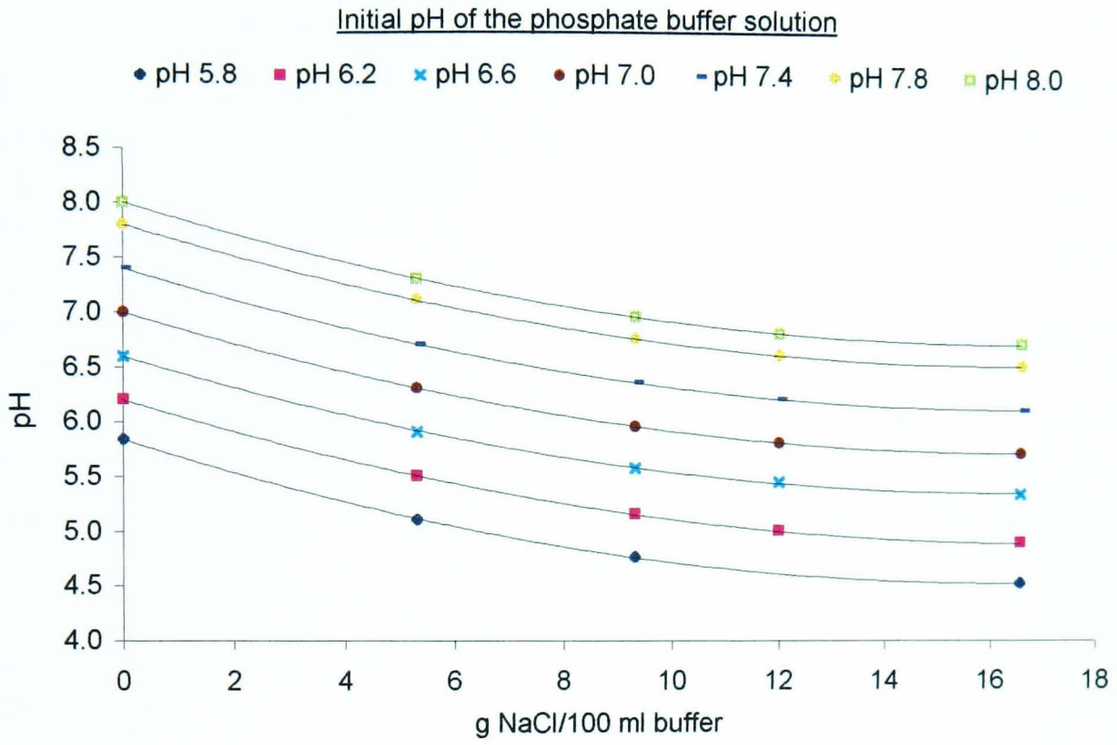


Figure 3.7 Effect of NaCl on the pH of a phosphate buffered solution. Values are the means of three replicates (standard deviations < 0.03).

3.8.3 Measurement of Niche Overlap Index (NOI)

With the results obtained from the carbon source utilisation profiles, a Niche Overlap Index (NOI) was calculated (Wilson and Lindow, 1995). This index expresses the coexistence or competition of different species in a nutritional niche and is defined as follows:

$NOI_{A/B} = NOI_{A} =$ N° CS utilised in common by species A and B / N° total CS used by species A

(NOI x 100) indicates the percentage of carbon sources utilised by species A that are also used by species B. Species A is referred to as the "target pathogen". NOI values > 0.9 indicate occupation of the same nutritional niche and values < 0.9 indicate the occupation of different niches (Wilson and Lindow, 1994).

NOI values are commonly presented in pairs as $NOI_{spp\ A/B}$ / $NOI_{spp\ B/A}$. Coexistence will appear when both NOI values are >0.9 while an individual of a pair of species with NOI < 0.9 will indicate the occupation of separate nutritional niches. Figure 3.8 illustrates the different types of species interactions considered.

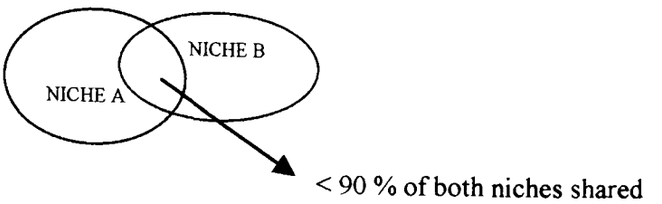
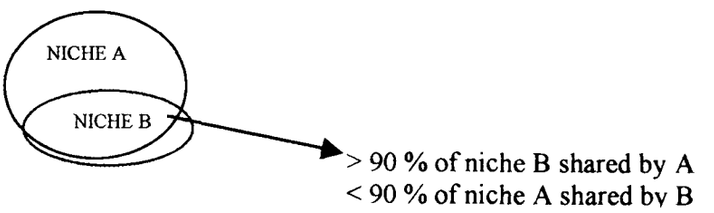
Occupation of separate niches		
$NOI_{A/B}$ <0.9	$NOI_{B/A}$ <0.9	
Occupation of same niche		Coexistence
$NOI_{A/B}$ >0.9	$NOI_{B/A}$ >0.9	
Occupation of same niche		Nutritional advantage of species A over B
$NOI_{A/B}$ < 0.9	$NOI_{B/A}$ > 0.9	

Figure 3.8 Types of interactions of paired species in relation with their relative NOI values.

3.9 EFFECT OF EXSITING AND ALTERNATIVE PRESERVATIVES ON HYDROLYTIC ENZYME ACTIVITY OF SPOILAGE FUNGI

3.9.1 Sampling and inoculation

The effect of environmental conditions and presence of preservatives on hydrolytic enzyme production patterns by the fungal species *Aspergillus ochraceus, Eurotium repens, Penicillium corylophilum, Penicillium roquefortii* and *Penicillium verrucosum* (strains M453 and PV3) was evaluated both *in vitro* and *in situ.* The effect of the preservative potassium sorbate and anti-oxidants BHA, PP and essential oils thyme and cinnamon was also studied in order to identify possible mechanisms of action against the spoilage moulds.

The enzyme activity on WFA and bread analogues was determined +/- preservatives at 25°C, pH 6 and three a_w levels, 0.97, 0.95 and 0.93. The effect of pH (4.5 and 6) was assayed at 0.95 a_w. Preservatives were added to 2% WFA at two different concentrations, 150 and 300 ppm (w/w) and their effect on enzyme production determined in combination with all a_w and pH treatments. On bread analogues, only one concentration, 300 ppm (w/w) at 0.97 a_w, pH 6 and 25°C was studied.

Using a sterile loop, fungal spores from a fresh culture on 2% WFA were harvested as previously detailed and the concentration of the spore suspensions adjusted to 10^{-7} spores ml^{-1}. Three plates per treatment and species were inoculated with 100µl of the spore suspension by spread plating to obtain an even coverage on the surface of the agar/bread. Three uninoculated plates for each treatment were kept and processed for enzyme production as a negative control.

Plates of 2% WFA were enclosed in polyethylene bags and bread analogues in containers with glycerol-water solutions, in order to maintain constant relative humidity values. Plates were incubated at 25°C for a period of 12 days.

3.9.2 Enzyme extraction

Three discs of agar/bread (20 mm diameter; 2 g sample approximately) of each replicate plate were taken using a cork borer after 3, 6, 9 and 12 days of incubation for the determination of enzyme production. The discs were placed into a plastic Universal bottle (25mm diameter, 30ml capacity) containing 4 ml of 10 mM potassium phosphate buffer (pH 7.2; Dawson *et al.*, 1987).

Bottles were shaken in an orbital shaker for 1h at 4°C. Three aliquots of each sample were decanted into 1ml eppendorff tubes and centrifuged on a bench microfuge at 13000 rpm for 15 minutes. Supernatants were carefully removed and stored at –20°C for enzyme activity analysis.

3.9.3 Measurement of enzyme activity

(i) Enzyme reaction

Total and specific activity of seven hydrolytic enzymes were measured using 4-nitrophenyl substrates (Sigma, Table 3.9). Enzymatic reactions were conducted in 96 well sterile microtitre plates. With the aid of a multi-channel micropippete (Gilson), 20µl of acetate buffer (25mM; Dawson *et al.*, 1987), 40 µl of enzyme extract (at room temperature and diluted as necessary) and 40 µl of nitrophenyl substrate were added to each well. The pH of the acetate buffer and concentration of p-nitrophenyl substrate solutions are shown in Table 3.9 (Jain and Lacey, 1991).

For each sample, a blank control well was used where no enzyme extract was added prior to the incubation process. Nitrophenyl substrates were the last component added to ensure in all wells a simultaneous start of the enzymatic reaction. Plates were sealed with an acetate cover to avoid evaporation, and incubated at 37°C for 60 min. After the incubation process, enzymatic reactions were stopped by the addition of 5µl of a 1M sodium carbonate solution (Sigma). Plates were left to reach room temperature for 3 minutes after which 40µl of enzyme extract were added to the blank control wells.

Table 3.9 Summary of the hydrolytic enzymes, substrates, substrate concentrations and pH of acetate buffer assayed.

Hydrolytic enzyme	*NP substrates*	*Concentration (mM)*	*Acetate Buffer pH*
β–D-Fructosidase	4-nitrophenyl-β-D-frucopyranoside	2	5
α-D-Galactosidase	4-nitrophenyl-α-D-galactopyranoside	4	5
β-D-Glucosidase	4-nitrophenyl-β-D-glucopyranoside	2	5
α–D-Mannosidase	4-nitrophenyl-α-D-mannopyranoside	4	5
β–D-Xylosidase	4-nitrophenyl-β-D-xylopyranoside	2	5
N-acetyl-β-D-Glucosaminidase	*p*-nitrophenyl-N-acetyl-β-D-Glucosaminide	2	4.2
N-acetyl-α-D-Glucosaminidase	*p*-nitrophenyl-N-acetyl-α-D-Glucosaminide	2	4.2

(ii) Measurement of total and specific enzyme activity

Total enzyme activity (nmols of 4-nitrophenol released h^{-1} g sample $^{-1}$) was measured using a multiscan microtitre plate reader (Dynex Technologies) as the increase in absorbance at 405 nm caused by the liberation of 4-nitrophenol (yellow colour) upon enzymatic hydrolysis of the substrate. The amount of p-nitrophenol liberated was calculated from a calibration curve where net absorbance at 405nm were plotted against p-nitrophenol concentrations. Calibration data were obtained by substituting the 40µl of enzyme extract in the reaction process by the same amount of p-nitrophenol (Sigma) solutions of known concentration. Two calibration curves were made for reactions carried out with pH 4.2 and 5 acetate buffers (Figures 3.9 and 3.10).

Specific enzyme activity (nmols 4-nitrophenol released h^{-1} µg $protein^{-1}$) was determined by dividing the total activity (expressed as nmols of 4-nitrophenol released h^{-1}) by the total amount of protein (µg) present in the enzyme extract.

(iii) Measurement of total protein content

Protein determination was carried out with a bicinchoninic acid kit for protein determination (BCA kit, Sigma) The kit consisted of bicinchoninic acid solution, copper (II) sulphate pentahydrate (4%) solution and Bovine Serum Albumin (BCA, 1mg ml^{-1}) as protein standard.

In each well of a 96-well microtitre plate, 10 µl of enzyme extract and 200µl of a solution made up with 1 part of copper (II) sulphate and 50 parts of bicinchoninic acid solution, were added. Wells with 10µl of 10mM phosphate buffer (pH 7.2) instead of the enzyme extract were used as blank control wells. Plates were sealed with an acetate cover to avoid evaporation and incubated at 37°C for 30 min. After the incubation process, plates were left to reach room temperature for 3 minutes.

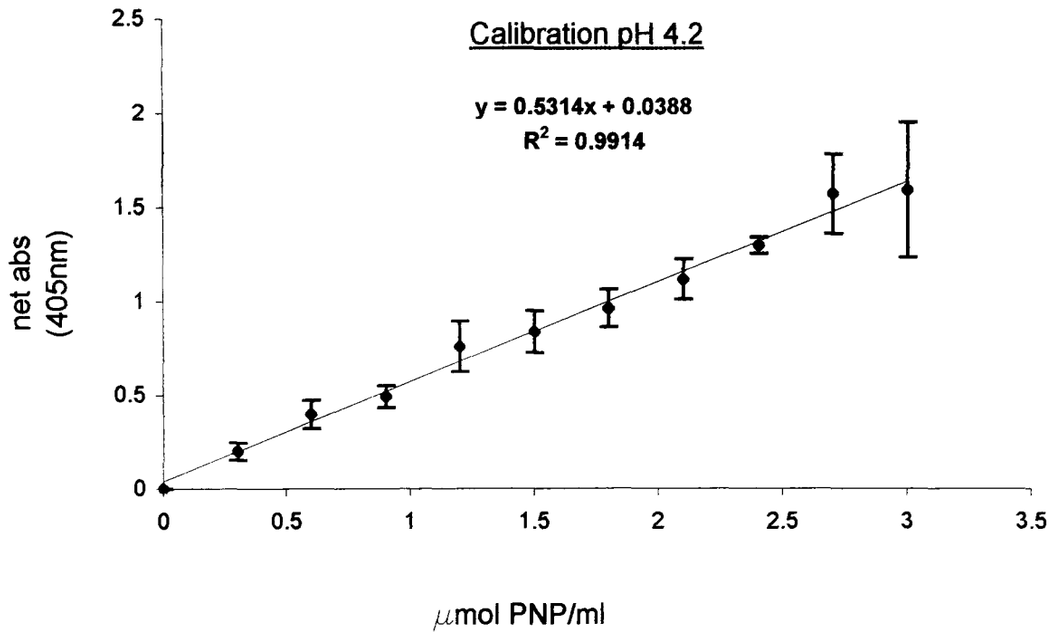


Figure 3.9 Calibration curve at pH 4.2 of p-nitrophenol (PNP) concentrations (μM ml^{-1}) against absorbance at 405nm. Each point are means of six replicates. Bars represent ± standard deviations.

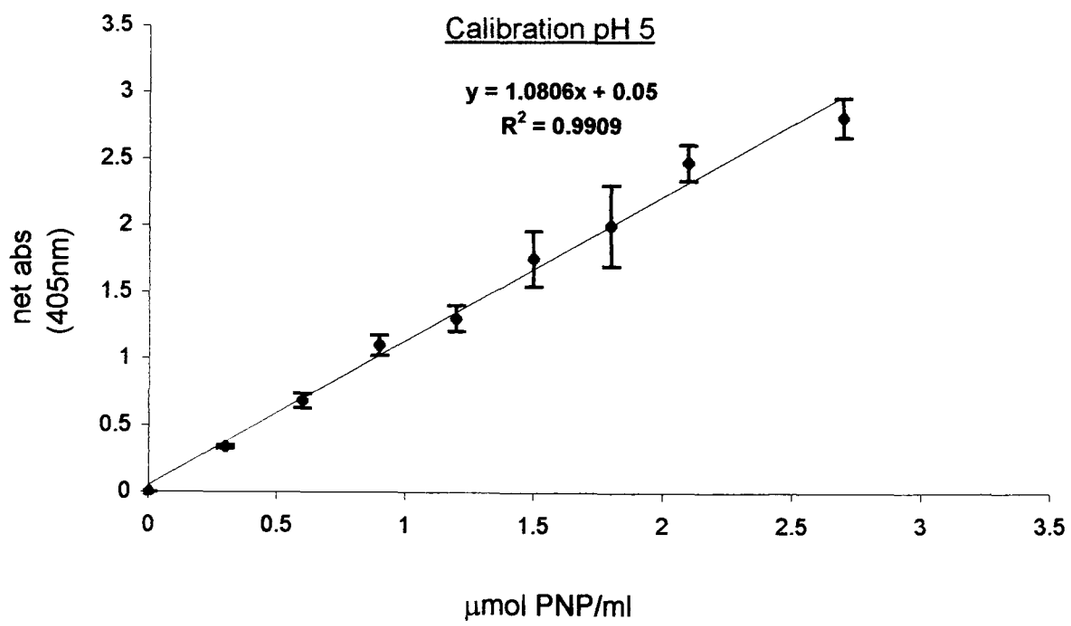


Figure 3.10 Calibration curve at pH 6 of p-nitrophenol (PNP) concentrations (μM ml^{-1}) against absorbance at 405nm. Each point are means of six replicates. Bars represent ± standard deviations.

Protein content was measured using a multiscan microtitre plate reader as the total absorbance at 530nm. The protein concentration in the enzyme extracts were calculated from the calibration curve of absorbance at 530 nm against known concentrations of BCA. The calibration curve was made following the SIGMA procedure No TPRO-562 (Figure 3.11).

3.10 STATISTICAL TOOLS

Data was statistically analysed by a general linear model procedure in order to study the significance of the factors assayed as well as the significance of their two, three and four-way interactions. Further analysis were carried out separately for each individual fungal species. Minitab 2.12 was used as the software for these analysis. Tables of results of the relevant statistical analysis are compiled in Appendix I.

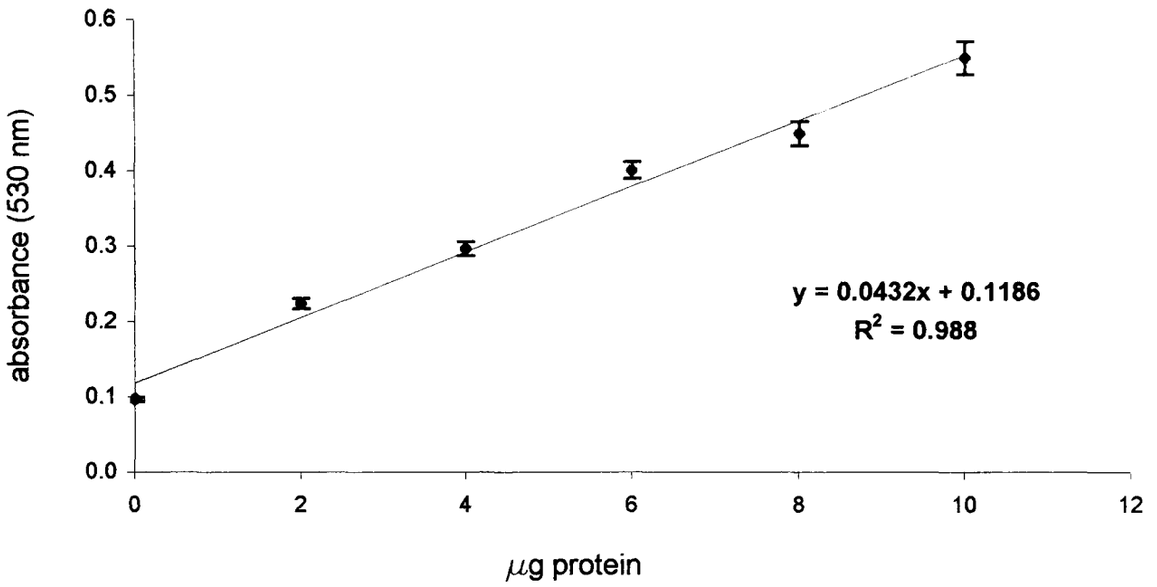


Figure 3.11 Calibration curve of μg of protein against absorbance at 530 nm. Points are means of six replicates. Bars represent ± standard deviations.

Chapter 4

RESULTS

4.1 EFFECT OF EXISITING PRESERVATIVES ON GROWTH OF SPOILAGE FUNGI ON 2% WHEAT FLOUR AGAR AT DIFFERENT ENVIRONMENTAL CONDITIONS.

The first set of experiments carried out in this project aimed to study the effect of existing preservatives (weak-acid) on *in vitro* growth of spoilage moulds at different environmental conditions. A wide range of a_w (0.80-0.95), pH (4.5-7.5) and temperature (15-20°C) was used representing intrinsic and extrinsic characteristics of different types of wheat-based bakery products which are currently preserved with salts of organic acids. The dose-effect of weak-acid preservatives on mould growth was evaluated focusing the study on the impact that the use of sub-optimal concentrations from the actually permitted level (3000 ppm; w/w) may have on mould growth and shelf-life of the product.

4.1.1 Effect of environmental factors on fungal growth

The single and combined effect of water activity (a_w), pH and temperature (T) on lag phase prior to growth and radial growth of common spoilage moulds was studied on 2% wheat flour agar (WFA). Different levels of a_w and pH were achieved as detailed in section 3.2.1. The actual values of a_w and pH for all prepared media were routinely measured. Maximum variations of ±0.009 and ±0.15 in a_w and pH levels respectively were observed.

The experimental error on measured growth rate values between both intra and inter experimental replicates was evaluated. Three replicate plates per experimental treatment (a_w x pH x T) were systematically measured. Additionally, a random small set of experiments were repeated again on a different day with newly prepared media and spore suspensions, in order to assess the reproducibility of results.

Overall, standard errors from 0.005 up to 0.15 mm radius day^{-1} were recorded, representing from 1 to 5% of the growth rate value. Growth rates obtained from replicated experiments varied from 0.04 to 0.5 mm radius day^{-1}($rate_1$-$rate_2$).

Variations from 0.01 to 0.08 mm radius.day^{-1} were generally observed between triplicate plates of the same experiment.

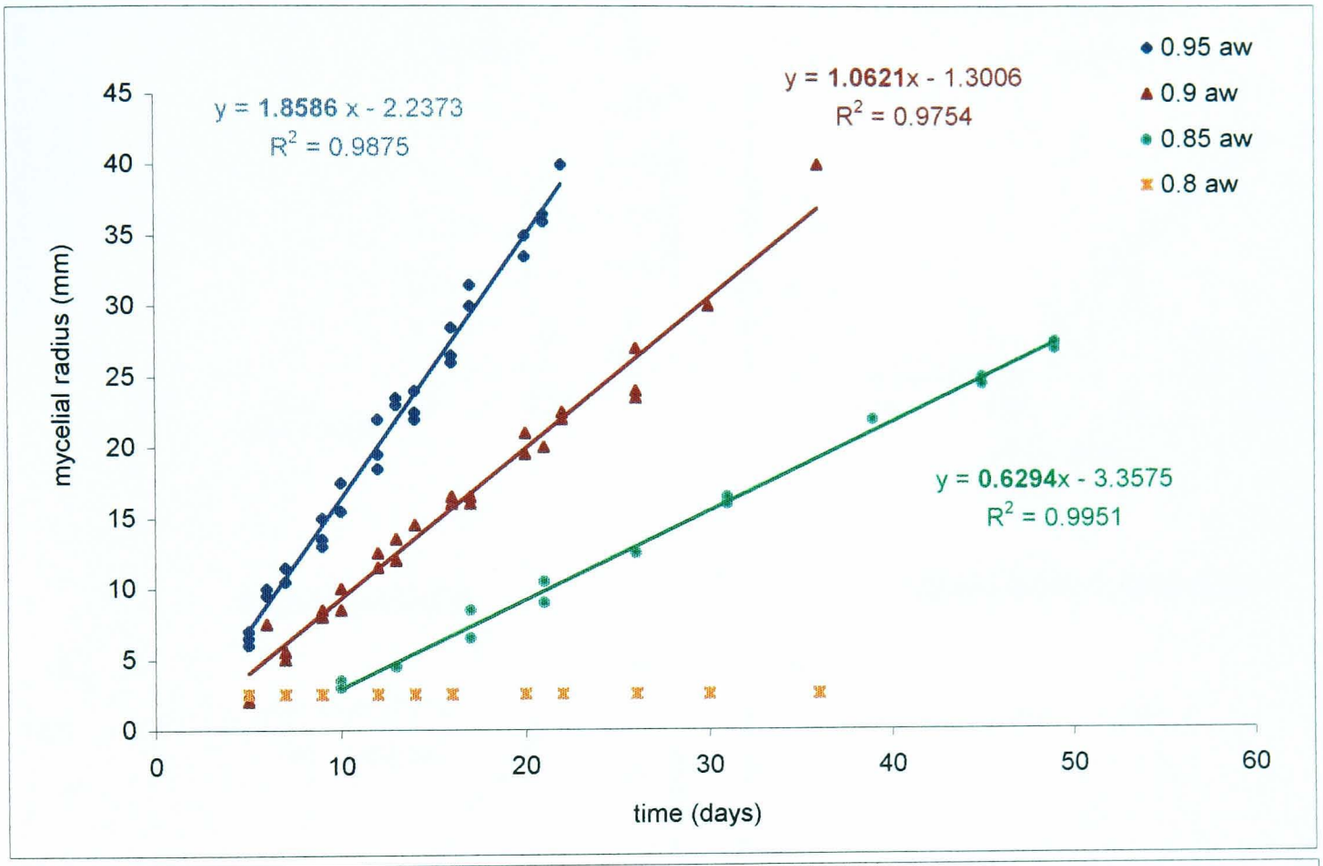
All individual environmental factors and most of their two way interactions exerted a significant effect ($p > 0001$) on both lag times and growth rates. Appendix I (Table I-1) summarises the results of a general linear model applied on the general effect of a_w, pH, species and T on lag times prior to growth and rate of colonisation.

(i) Water activity

Figure 4.1 illustrates the mycelial radius extension over time of colonies of *A.ochraceus* at the different a_w levels, pH 7.5 and 25°C. This figure also exemplifies how lag phases and growth rates are calculated from the linear regression obtained from plotting the colony radius of all replicates against time. Furthermore, in Figure 4.2 the effect of a_w on lag times prior to growth and radial growth rates on cultures of *P.corylophilum*, *E.repens* and *C.herbarum* on WFA is illustrated at 25°C and pH 6.

Water activity was the most significant parameter that influenced the growth of all species studied (see Appendix I, table I-1). In general, for all pH levels and temperatures tested, lag times increased and growth rates decreased significantly ($p<0.001$) as the a_w level of the substrate was reduced.

Maximum growth rates values were found at the highest a_w tested, 0.95. *E.repens* and *A.ochraceus* showed fastest with rates $\geq$ 2.5mm day^{-1} and lag phases shorter than 4 days at 25°c and pH <7.5. In contrast, regardless of pH and T levels, none of the species was able to grow at the lowest a_w level tested (0.80) during the period of time of the experiment (30 days). Overall, *C.herbarum* was most sensitive, only growing at $\geq$ 0.95 a_w.



Linear Regression equation	Y = A X + B
Lag Time	(2.5 - B) / A
Growth rate	A

Figure 4.1 Effect of water activity (a_w) on mycelial radial extension *Aspergillus ochraceus* growing on 2% wheat flour agar, pH 7.5 and 25°C.

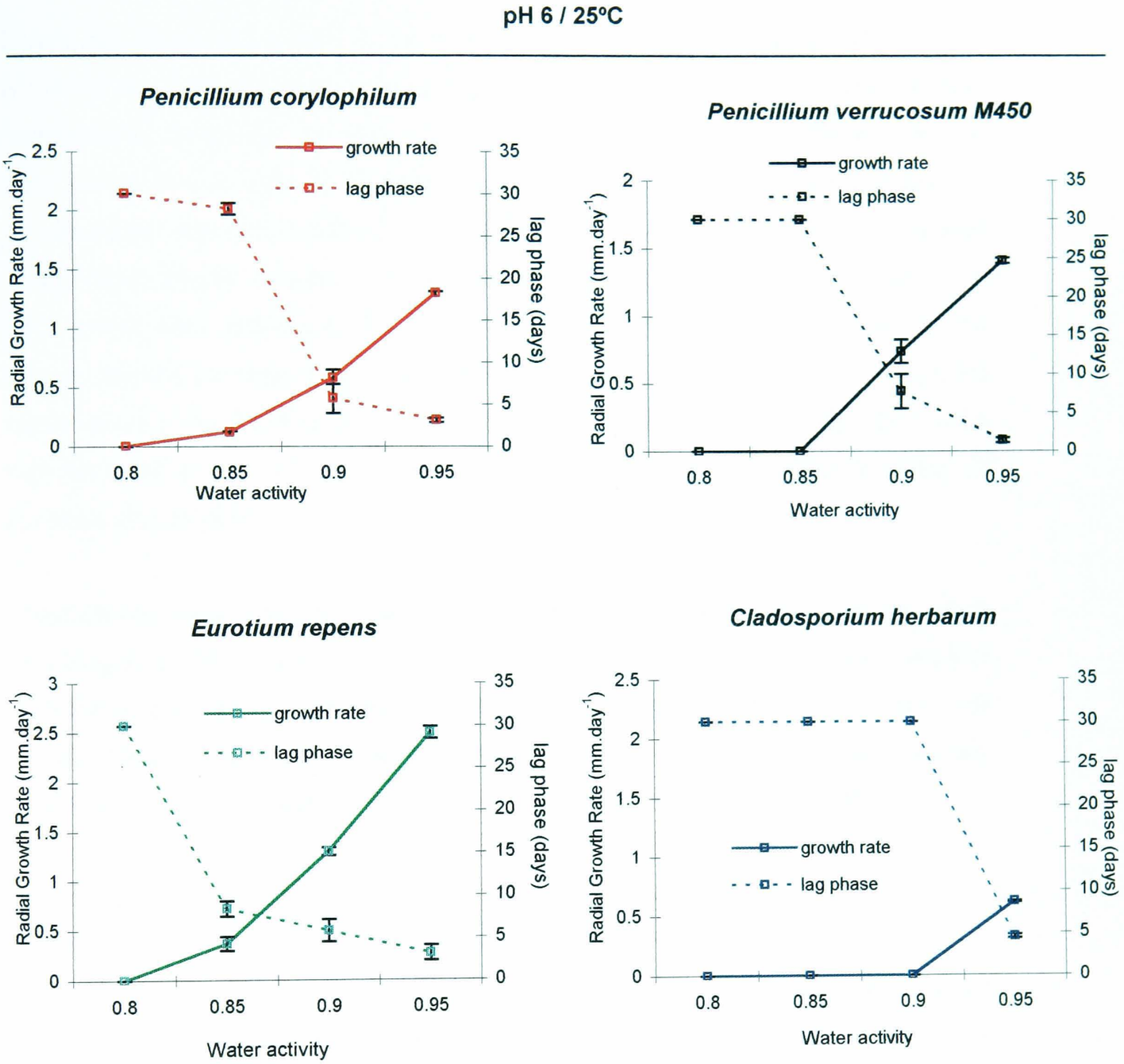


Figure 4.2 Effect of water activity on lag times (days) and growth rates (mm day^{-1}) of *Penicillium corylophilum, Eurotium repens, Penicillium verrucosum (strain M450)* and *Cladosporium herbarum* growing on 2% wheat flour agar, pH 6 and 25°C (bars represent ± standard deviations from the mean of three replicates).

(ii) pH

Figure 4.3 shows an example of the effect of pH on lag phases and growth rates of *A.ochraceus*, *P.verrucosum* (strain M453) and *E.repens* at 0.95 a_w and different temperatures. Generally, for each a_w level at both temperatures, fungal growth was faster as the pH was lowered. Optimum growth was observed at pH 4.5 and 6. However, no statistically significant differences were observed between pH 4.5 and 6 for growth of any of the species studied. The effect of increasing pH values from pH 6 to 7.5 on both growth rates and lag phases was significant only at 25°C and for some of the species studied. *E.repens* and *A.ochraceus* grew significantly slower with longer lag phase values at pH 7.5 than at pH 4.5 ($p<0.05$). On the other hand, pH did not exert a significant effect ($p>0.05$) on either lag phases or growth rates of both strains of *P.verrucosum* studied.

Although in general terms, lag phases and growth rates were affected to a similar extent by changes in pH, in some cases, the effects on lag phase duration were slightly different to those for growth rates. For instance, while growth of *P.corylophilum* was not significantly affected by changes in pH ($P>0.05$), the lag phases were markedly longer as pH was increased from 4.5 to 6 ($p<0.05$) and from 6 to 7.5 ($p<0.001$).

(iii) Temperature

In Figure 4.4 the effect of temperature on lag phases and growth rate values of all species studied is illustrated at pH 4.5 and 0.90 a_w. Overall, lag phases were significantly longer and growth slower ($p<0.05$) at 15 than at 25°C. *A. ochraceus* was the most sensitive species to temperature with more than 50% growth rate reduction and increase in lag phases of 2 days at 0.95a_w and up to 27 days at a_w 0.85. For other species examined, between 24 to 100% growth reduction was observed. For *E.repens*, although lag phases were affected by a temperature reduction ($p<0.001$) with an increase of between 5.6 to 18.2 days, the differences in growth rates were not significant ($P>0.05$). Lag phases of *Penicillium* isolates were affected to a lesser extent with increases of 0.5-2.5 days at 0.95 a_w and between 3.5 and 7.9 days at 0.90-0.85 a_w.

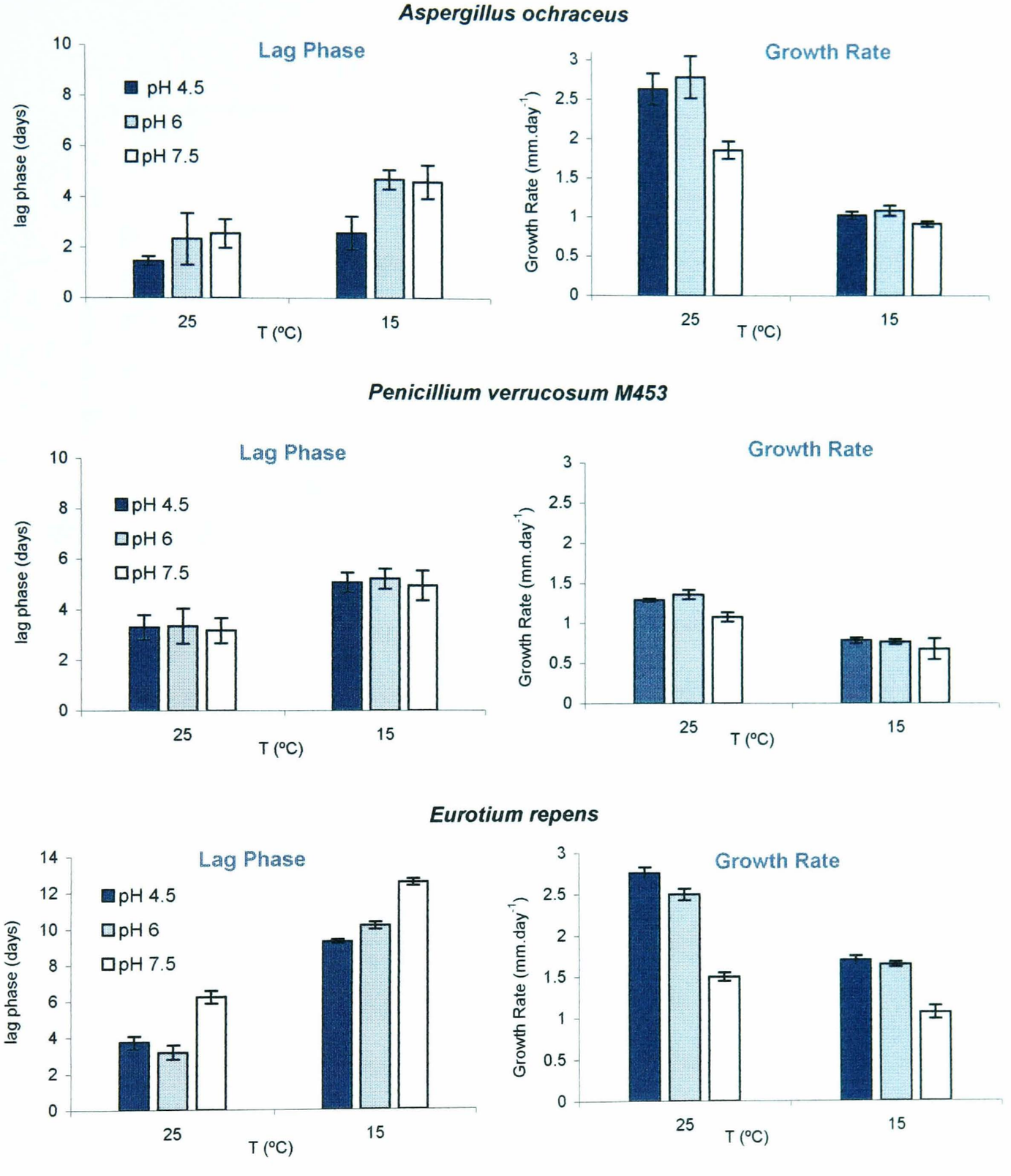


Figure 4.3 Effect of pH on lag phases (time, in days) and growth rates (mm day^{-1}) of *Aspergillus ochraceus*, *Penicillium verrucosum* (M453) and *Eurotium repens* growing on 2% wheat flour agar at 15 and 25°C and 0.95 a_w (bars indicate ± standard deviations from the mean of three replicates)

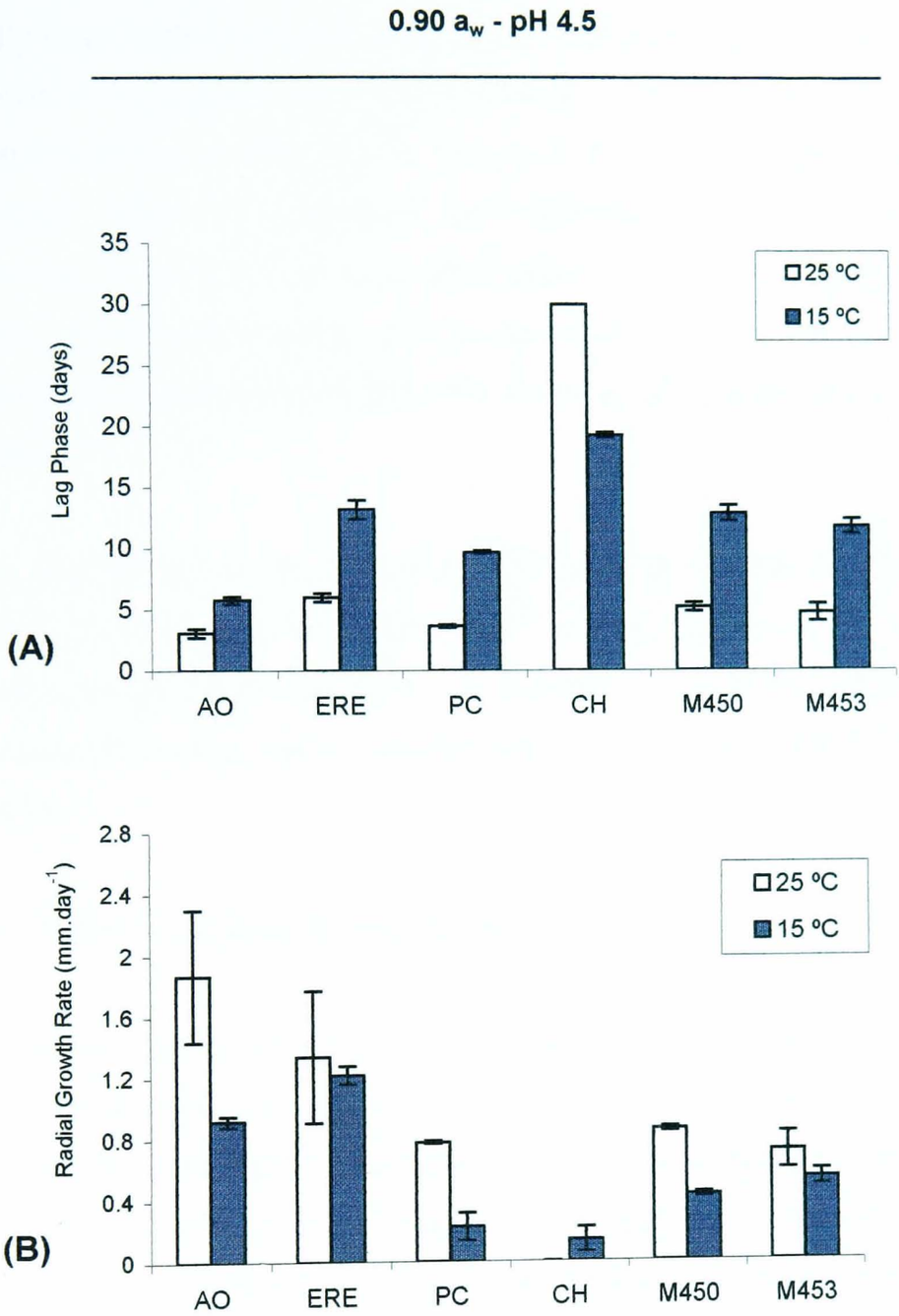


Figure 4.4 Effect of temperature on **(A)** lag phases (days) and **(B)** growth rates (mm day^{-1}) of *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicilium corylophilum* (PC), *Cladosporium herbarum* (CH) *and Penicillium verrucosum* (strains M450 and M453) growing on 2% wheat flour agar, pH 4.5 and 0.90 a_w (bars indicate ± standard deviations from the mean of three replicates)

(iv) Combined effect of a_w, pH and T

Interactions between a_w and temperature had a significant effect ($p<0.05$) on fungal growth. The a_w range over which the fungal species were able to grow appeared to be narrower at low temperature. In fact, at 15°C no species tested were able to grow at $a_w<0.85$. When the temperature was increased to 25 °C all fungal species except *C.herbarum* grew at 0.85 a_w at some extent. Furthermore, a reduction of temperature had a more marked effect on lag phases and growth rate at lower a_w level. No significant interactions ($p>0.05$) were recorded between pH and a_w or T (see Appendix I, Table I-1).

The combination of the environmental conditions studied that best supported fungal growth was 0.95 a_w, pH 4.5- 6 and 25°C. At these conditions, growth rate values for all species except *C.herbarum* were >1.3 mm day^{-1} with <6 day lag phase prior to growth. Overall, of the fungi tested, *Eurotium* spp achieved fastest colonisation rates of up to 2.8 mm day^{-1}.

4.1.2 Effect of existing chemical preservatives

The effect of sub-optimal concentrations and environmental factors on the antimicrobial effect of the existing preservatives, sodium benzoate, calcium propionate and potassium sorbate was evaluated in comparison with the actual maximum permitted level (3000 ppm; w/w). Two serial log dilutions of the maximum level were used (300 and 30 ppm). The salts were added to the substrate by homogenising them with the rest of the solid ingredients as it is practically incorporated into the food product.

The effect of environment (a_w x pH x T combinations) on the inhibitory effect of the preservatives was firstly screened using potassium sorbate. The lowest a_w level (0.80) was not tested in subsequent experiments with sodium benzoate and calcium propionate as no growth was observed in any of the treatments carried out with potassium sorbate at this a_w level.

The addition to the media of 3000 ppm of all three salts of organic acids had a significant effect on the final pH of the media. Maximum increases of pH of 0.26 were recorded. No marked effect was observed when sub-optimal concentrations were used.

(i) Effect of the concentration of preservative

Figure 4.5 shows the effect of different concentrations of potassium sorbate on growth rates of *A.ochraceus* at different temperatures, a_w and pH levels.

In all environmental conditions, doses of 3000 ppm (w/w) of all three salts were effective at inhibiting mould growth, except at the highest pH level, 7.5. At the lowest pH (4.5) complete inhibition (100%) was observed regardless of the temperature or a_w. More detailed studies were subsequently carried out to compare the efficacy of log dilutions of the maxim dose of preservative in relation to other environmental factors.

The use of concentrations < 3000 ppm were not always effective. Although some species were still inhibited by lower concentrations of preservative, other species did not follow the same pattern. Figure 4.6 depicts the % inhibition of growth of *P.verrucosum* strain M453 in the presence of the different concentrations of potassium sorbate, calcium propionate and sodium benzoate at three pH levels, 25°C and 0.90 a_w.

Interestingly, statistically significant stimulation ($p<0.05$) of growth of some species was observed when sub-optimal concentrations of organic acids were used at pH 6 and 7.5. This effect was particularly noticeable for the ochratoxigenic species *P. verrucosum*. For instance, in Figure 4.6, significant growth stimulation (negative inhibition values) can be observed, particularly in the presence of sodium benzoate. However, enhancement of growth was not always related to the decrease in the lag phase prior to growth. In fact, although low doses (30 ppm) of sodium benzoate, for example, did significantly stimulate ($p>0.05$) growth of *A.ochraceus* at 0.95 a_w and pH 4.5, an increase of 2 days in the lag phase duration was recorded.

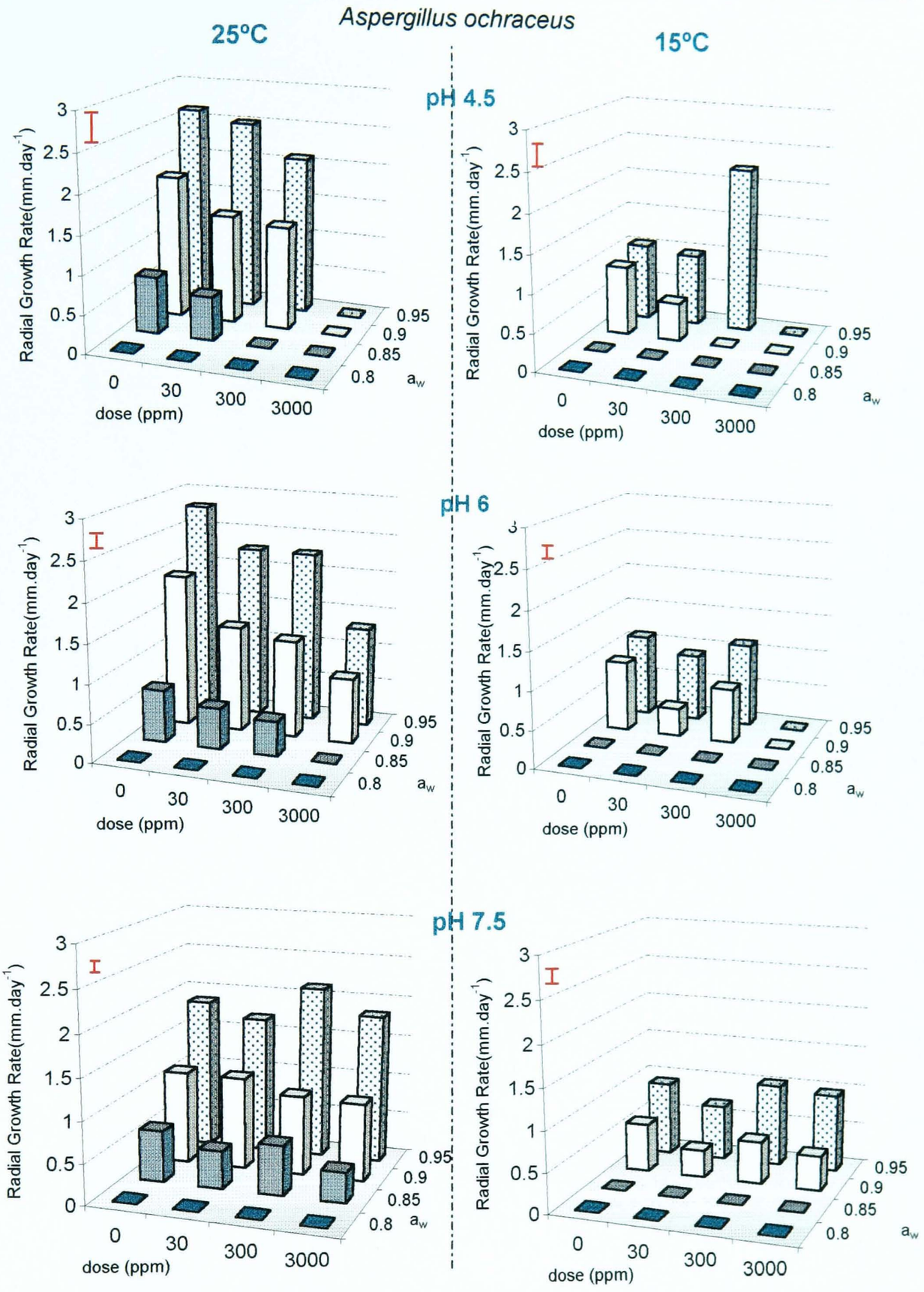


Figure 4.5 Effect of water activity (a_w) and dose of potassium sorbate (ppm) on the growth rate of *Aspergillus ochraceus* growing on 2% wheat flour agar at 25°C and different pH levels. Bars represent Least Significant Differences (LSD) at $p < 0.05$.

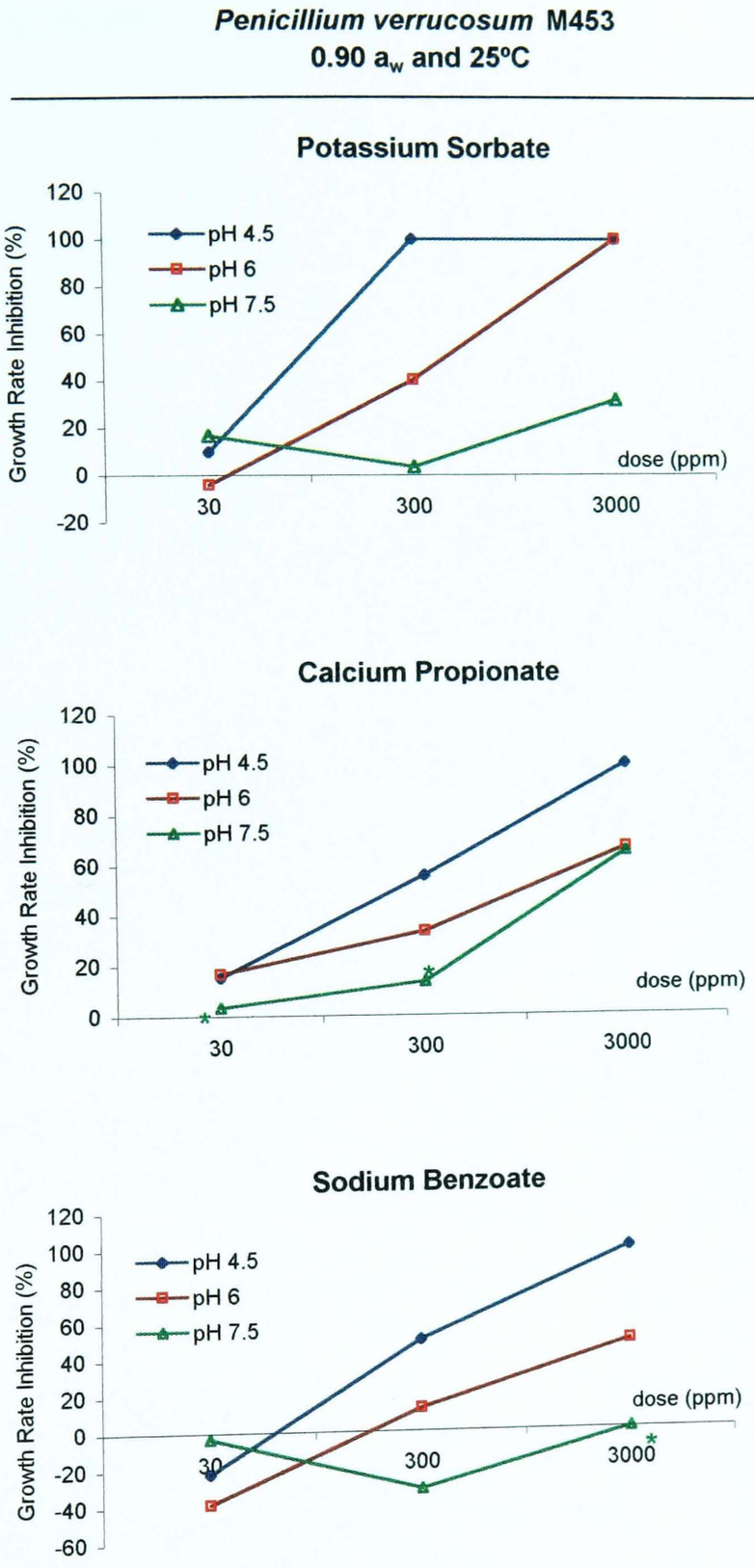


Figure 4.6 Effect of dose and pH on percentages of growth rate inhibition (%) of *Penicillium verrucosum* M453 growing on 2% wheat flour agar, 25°C and 0.90 a_w, in the presence of potassium sorbate, calcium propionate and sodium benzoate.

(ii) Effect of environmental factors on preservative efficacy

Figure 4.7 illustrates the effect of a_w and pH on the inhibitory effect of 3000 ppm of calcium propionate in cultures of *E.repens* and *A.ochraceus*. Table 4.1 shows the effect of preservative type and concentration on mould-free times at 25°C and at the different combination of a_w and pH studied. pH was the environmental factor that most significantly determined the antimicrobial action of all three acid preservatives ($p<0.001$). The effectiveness of all preservatives in increasing lag phases and reducing mould growth was highest at pH 4.5 and decreased as the pH was increased. This pattern was observed when the highest concentration of the acid was used. When the preservative was used at lower concentrations, this effect was not always significant.

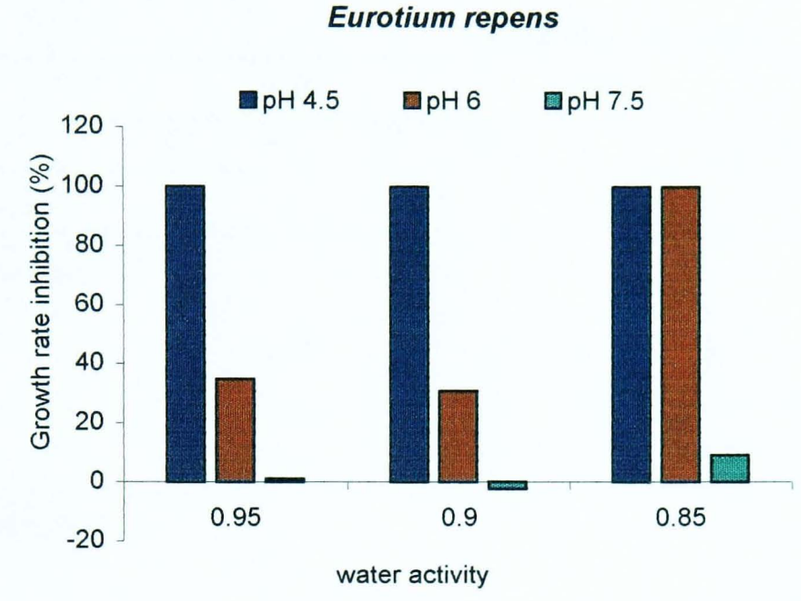
At pH 4.5 and for all three preservatives, sub-optimal concentration of 300 ppm was still effective at controlling, to some extent, growth of all species studied. Complete inhibition of *Penicillium* and *Cladosporium* isolates was observed with 3000 ppm potassium sorbate regardless of a_w or temperature. For *A.ochraceus* and *E.repens* the above only occurred at $a_w \leq 0.85$ (data not shown).

Inhibition of fungal growth appeared to be greater as the water activity of the substrate was reduced. Again, this effect was only consistent for the higher concentration of preservative, 3000 ppm. Overall, at 15°C similar patterns were obtained.

(iii) Effect of preservative type used

Figure 4.8 compares the relative inhibition of growth achieved with 3000 ppm potassium sorbate, calcium propionate and sodium benzoate at $0.95a_w$ and pH 6 for cultures of *P.corylophilum* and two strains of *P.verrucosum*. Overall, no statistically marked differences in the inhibitory effect between calcium propionate and sodium benzoate were observed ($p>0.05$). However, potassium sorbate showed slightly better control of fungal growth at low pH levels (4.5-6) than sodium benzoate and calcium propionate particularly for *Penicillium* species ($p < 0.05$).

CALCIUM PROPIONATE
3000 ppm (w/w)



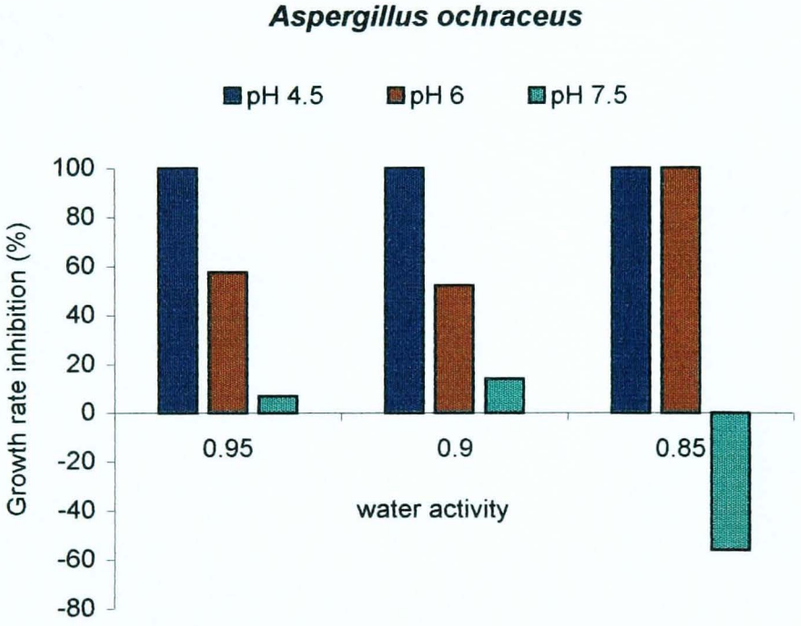


Figure 4.7 Effect of water activity and pH on the percentage growth inhibition (%) of cultures of *Eurotium repens* and *Aspergillus ochraceus* growing on 2% wheat flour agar in the presence of 3000 ppm (w/w) of calcium propionate.

Table 4.1 Effect of type and dose of preservative on minimum period of time (days) in which no growth was observed for any of the species studied (mould-free time), at 25°C and different water activity (a_w) and pH levels.

	Mould-free time (days)								
a_w	**0.95**			**0.90**			**0.85**		
pH	**4.5**	**6**	**7.5**	**4.5**	**6**	**7.5**	**4.5**	**6**	**7.5**
No preservative	1.5	1.4	2.5	3.1	3.8	4.3	7.2	7.6	6.3
Potassium sorbate (ppm; w/w)									
3000	30	17.2	3.2	30	18.8	4.7	30	30	9.9
300	8.9	2.2	2.5	12.3	3.6	1.1	30	1.5	1.4
30	1.9	1.4	1.8	2.7	1.7	1.3	1.9	7.9	9.5
Calcium propionate (ppm; w/w)									
3000	30	3	1.3	30	11.5	1.3	30	30	8.5
300	3	1.3	1.2	5.2	2.7	1.1	10.8	3.6	5.1
30	2.3	0.2	0.9	0.4	1.5	0.9	5.5	4.6	2.3
Sodium benzoate (ppm; w/w)									
3000	30	1.7	1.35	30	3.4	0.5	30	14	6
300	3.3	0.65	1.25	6.6	2.0	1.25	11.3	5	5.9
30	1.0	1.75	0.55	1.7	0.9	0.7	6.7	6.5	7.15

3000 ppm at pH 6 and 25°C

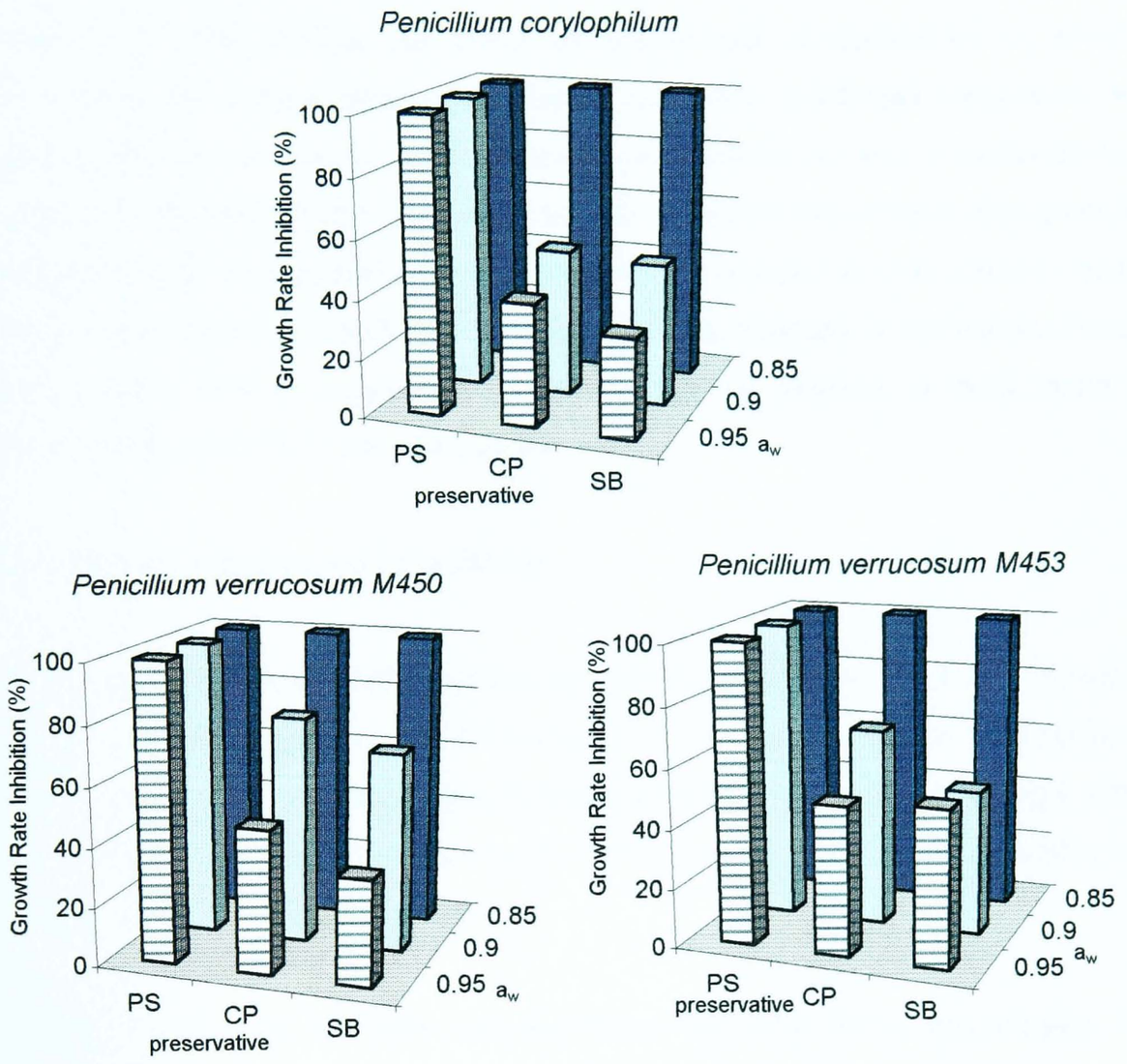


Figure 4.8 Growth rate inhibition (%) of *Penicillium corylophilum* and *Penicillium verrucosum* (strain M450 and M453) growing on 2% wheat flour agar at 25°C and pH 6 in the presence of 3000 ppm (w/w) of potassium sorbate, calcium propionate and sodium benzoate at three different a_w levels. All inhibition values are statistically significant when compared to untreated controls ($p < 0.05$).

4.2 EFFECT OF EXISITING PRESERVATIVES ON GROWTH OF SPOILAGE FUNGI ON BREAD ANALOGUES AT DIFFERENT ENVIRONMENTAL CONDITIONS.

Similar to *in vitro* studies, the effect of sub-optimal concentrations of existing preservatives, and the potential application of novel/new antifungal compounds was evaluated on bread analogues. All experiments were carried out over a narrower range of a_w (0.97-0.93) and pH (4.5-6) in order to better simulate the intrinsic characteristics of bread products. Species *Penicillium roquefortii* was included in these studies because of its incidence in mould spoilage of sourdough breads common in continental Europe (Spicher 1984 cited in Legan & Voysey, 1991). In addition, a third strain of *P.verrucosum* (strain PV3) was also studied.

4.2.1 Effect of environmental conditions

Overall, results followed similar patterns as previously reported for WFA. However, contrary to what observed *in vitro*, pH had a highly significant effect on both lag times and growth rates. On bread analogues, all species grew significantly faster at pH 6 than pH 4.5 with and without preservatives ($P<0.001$) except for *P.roquefotii* which had faster growth at the lower pH level, 4.5.

Growth rates decreased in a statistically significant manner as the a_w was reduced. The effect of a_w on lag phases was species dependent with *A.ochraceus, P.corylophilum* and *P.verrucosum* (strain PV3) the only species significantly affected. *E.repens* and *A.ochraceus* grew fastest with colonisation rates of up to 9 and 3 mm day^{-1} respectively at 0.97 a_w and pH 6, with no preservatives added. At the same conditions, *Penicillium* and *Cladosporium* isolates grew at about 2 mm day^{-1}.

Table I-3 in Appendix I summarises the analysis of variance of the effect of a_w, pH and species on lag phases prior to grow and growth rates..

4.2.2 Effect of existing preservatives

Potassium sorbate (most effective of the existing preservatives in *in vitro* mould control) and calcium propionate (actually used in bread preservation) were used at the optimum concentration of 3000 ppm (w/w) and a sub-optimal concentration of 300 ppm. Following the results obtained on WFA, preservative doses of 30 ppm were considered impractical and therefore not used for *in situ* experimentation. Preservative effectiveness was again evaluated at different environmental conditions.

Table 4.2 summarises for all species, the effect of pH on increases in lag phase and growth rate reductions achieved with 3000 ppm of potassium sorbate and calcium propionate at $0.97a_w$ and 25°C. In Figure 4.9 the combined effect of a_w, and dose of potassium sorbate on mould growth of all species at pH 4.5 is shown. Plate 4.1 shows colonies of *P.verrucosum* (strain PV3) on bread analogues with and without potassium sorbate and calcium propionate, at 25°C, 0.97 a_w and pH 6 after 14 days incubation.

Complete inhibition of all species, except *P.roquefortii* at 0.97 a_w, was only achieved by 3000 ppm potassium sorbate and calcium propionate at pH 4. At pH 6, with the exception of *C.herbarum*, preservative doses of 3000 ppm led to a maximum of 64% inhibition of growth. Under these conditions, although the rate of colonisation of *P.roquefortii* at the highest level of a_w (0.97) was not significantly affected by the addition of preservative, a significant 14 day increase in lag phase was observed in the presence of 3000 ppm of calcium propionate at pH 6.

The use of preservatives at the sub-optimal doses of 300 ppm was generally ineffective at controlling mould growth. Moreover, some growth stimulation was observed at both pH levels particularly for the *Penicillium* isolates. Figure 4.10 illustrates the effect of dose of calcium propionate on growth rate of all species studied at pH 6 and at different a_w levels.

Table 4.2 Effect of pH on percentage of growth rate inhibition and on lag phase increase of all species growing on bread analogues at 0.97 a_w and in the presence of 3000 ppm of potassium sorbate (PS) and calcium propionate (CP). Numbers in bold indicate values statistically significant when compared to untreated control ($p < 0.05$).

Growth Rate Inhibition (%)				
Preservative	PS		CP	
pH	*4.5*	*6*	*4.5*	*6*
A.ochraceus	**100**	**32.7**	**100**	**32.7**
C.herbarum	**100**	**100**	**100**	**63.8**
E.repens	**100**	**59.5**	**100**	**57.4**
P.corylophilum	**100**	**42.6**	**100**	**30.8**
P.roquefortii	54.6	-14.6	-36.6	48.3
P.verrucosum M450	**100**	**36.8**	**100**	**17.2**
P.verrucosum M453	**100**	**48.8**	**100**	**8.43**
P.verrucosum PV3	**100**	**60.5**	**100**	22
Increase of Lag Phase Duration (days)				
A.ochraceus	**29.4**	**0.12**	**29.4**	-0.02
C.herbarum	**16.8**	**24.6**	**16.8**	5
E.repens	**26.2**	3	**26.2**	3.14
P.corylophilum	**26.3**	3.5	**26.3**	3
P.roquefortii	8.5	7.7	2.43	**14.4**
P.verrucosum M450	**27.8**	2.5	**27.8**	0.2
P.verrucosum M453	**28.1**	0.8	**28.1**	0.1
P.verrucosum PV3	**29.2**	2.4	**29.2**	1.24

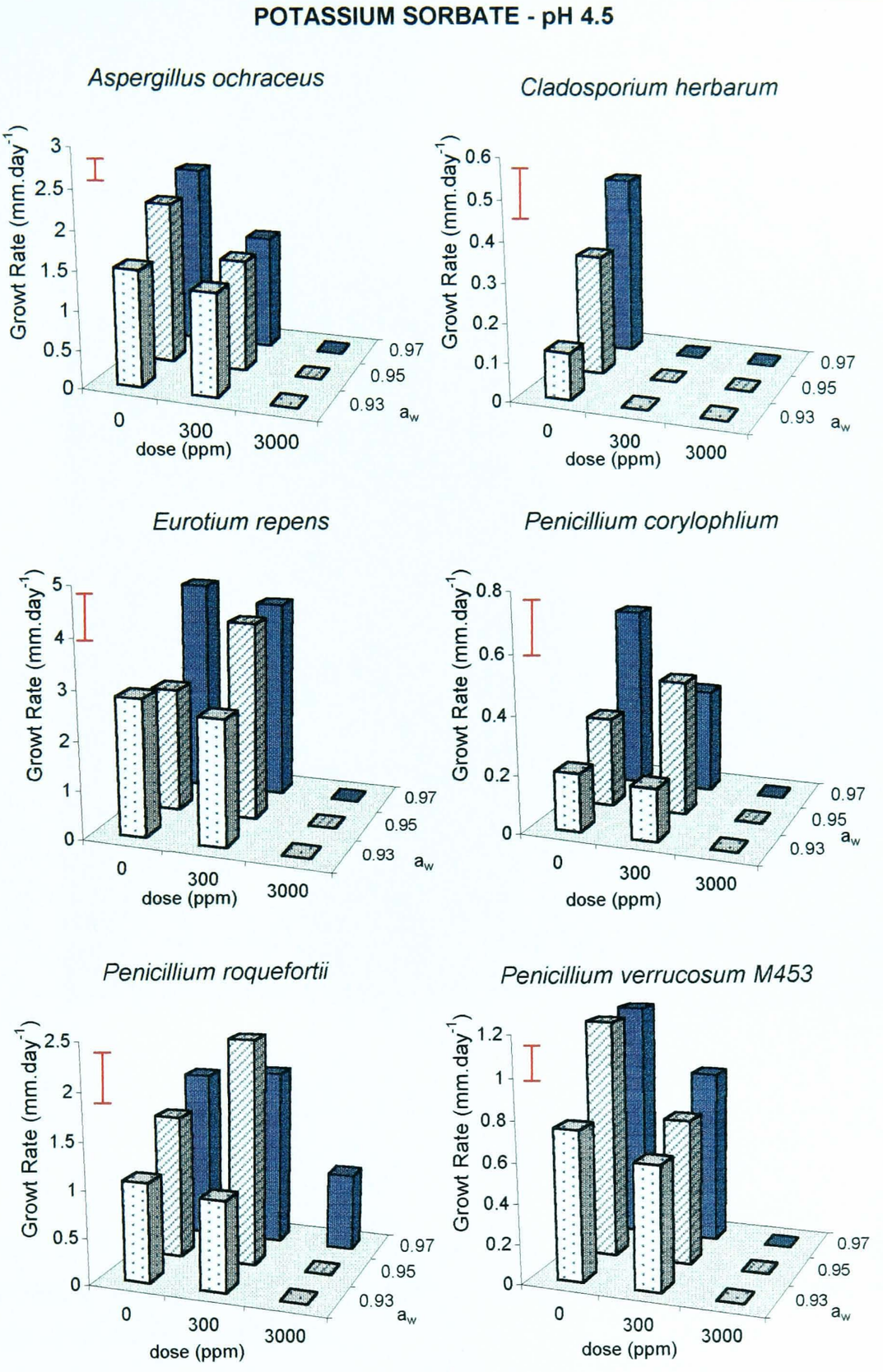


Figure 4.9 Effect of water activity (a_w) and dose of potassium sorbate on growth rates (mm day^{-1}) of *Aspergillus ochraceus, Cladosporium herbarum, Eurotium repens, Penicillium corylophilum, Penicillium roquefortii* and *Penicillium verrucosum* (strain M453) growing on bread analogues at pH 4.5 and 25°C. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

Penicillium verrucosum **(strain PV3) at 0.97 a_w pH 6 and 25°C**

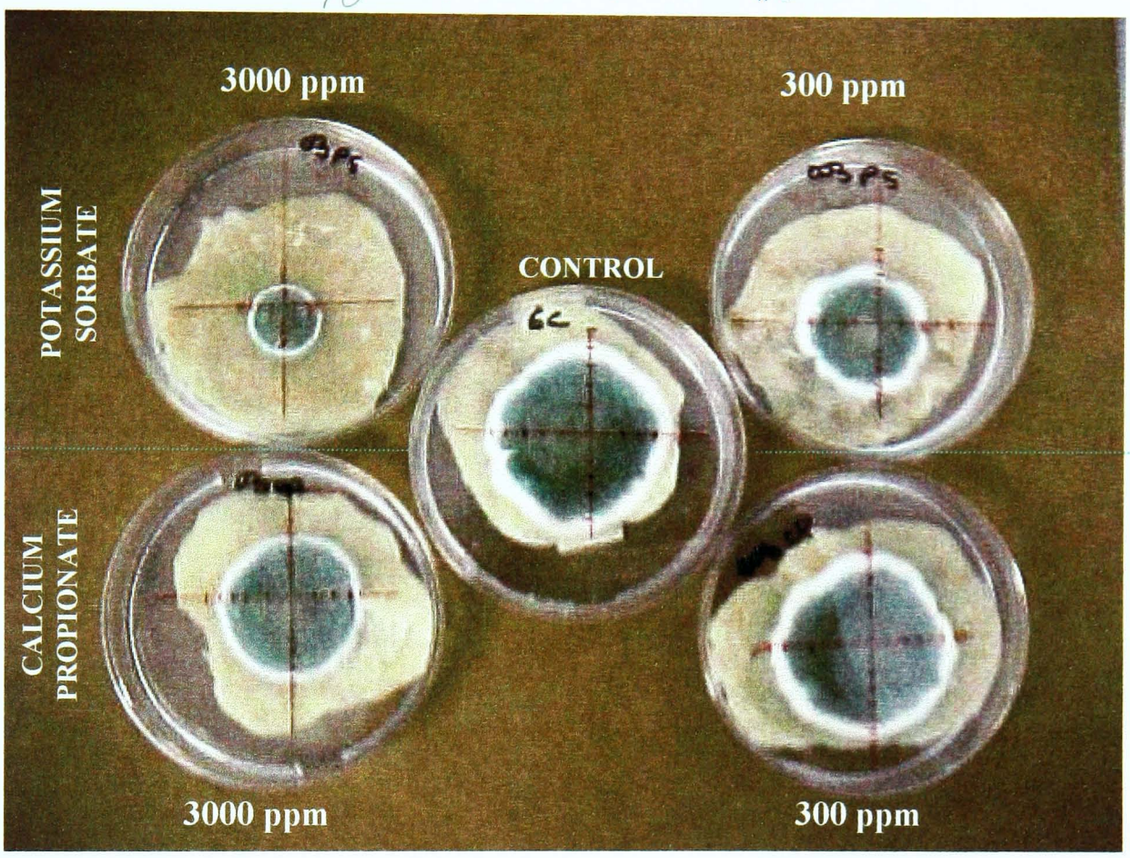


Plate 4.1 Colonies of *Penicillium verrucosum* (strain PV3) on bread analogues with and without potassium sorbate and calcium propionate (at 3000 and 300 ppm (w/w)), at 25°C, 0.97 a_w and pH 6 after 14 days of incubation.

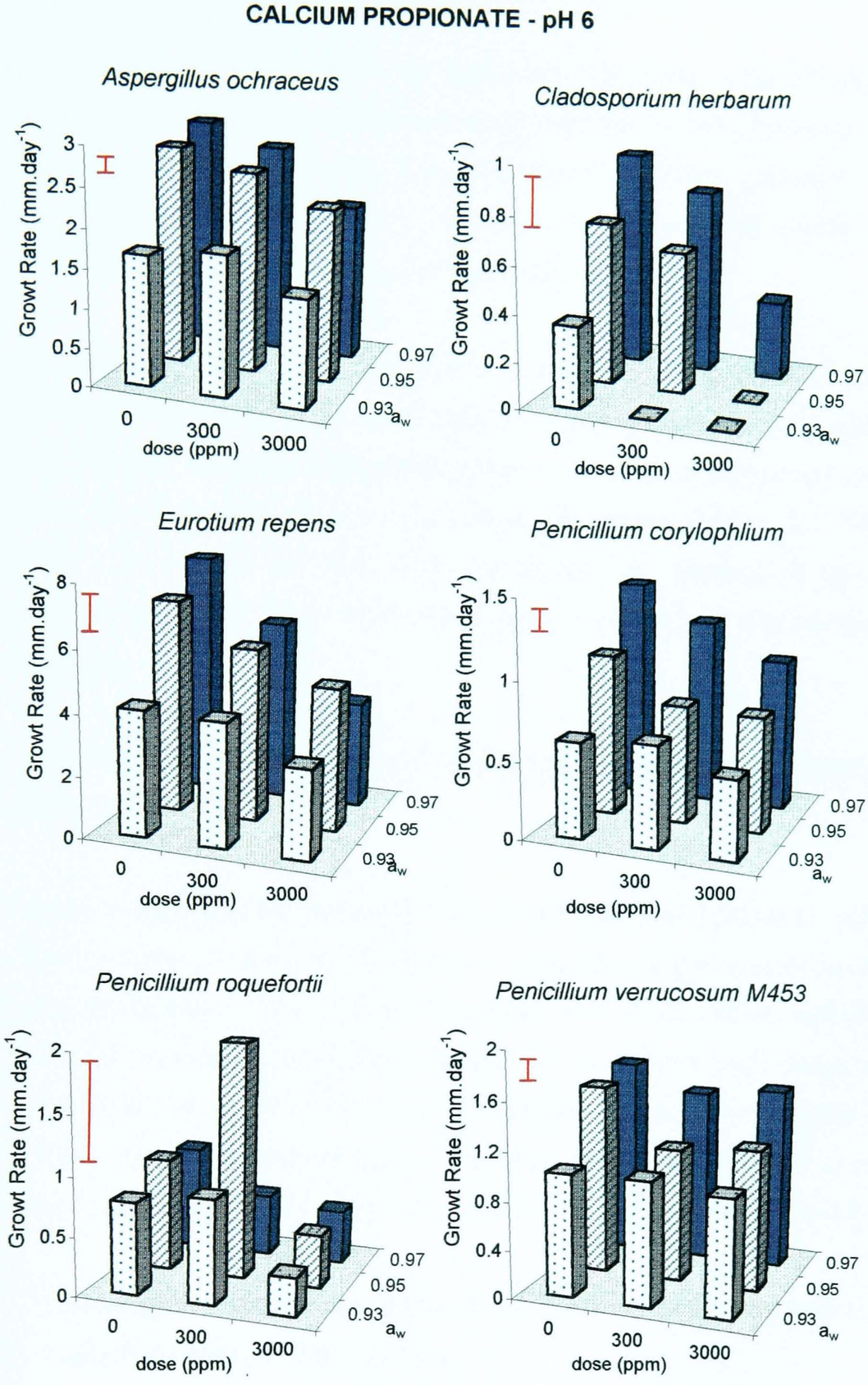


Figure 4.10 Effect of water activity (a_w) and dose of calcium propionate on growth rates of *Aspergillus ochraceus, Cladosporium herbarum, Eurotium repens, Penicillium corylophilum, Penicillium roquefortii* and *Penicillium verrucosum* (strain M453) growing on bread analogues at pH 6 and 25°C. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

Similar to what observed on WFA, no significant differences in the efficacy between potassium sorbate and calcium propionate were observed ($p<0.05$). However, potassium sorbate appeared to be statistically more effective in inhibiting growth of strains of *P.verrucosum.* Overall, *C.herbarum* was again the species most sensitive to both preservatives , and only grew in the presence of 300 ppm at pH 6.

All individual factors; a_w, pH, and dose of preservative markedly affected both lag phases and growth rates of all species studied ($p<0.05$). In the presence of organic acids, dose and pH were the factors with greater influence on both lag phases and growth rate. Generally, the preservative effect was greater at the lowest pH value, 4.5. Table I-4 in Appendix I summarises the results of the analysis of variance of the effect of preservation and environmental factors, and their two and three way interactions, on mould growth.

4.3 EFFECT OF ANTIOXIDANTS AND ESSENTIAL OILS ON GROWTH OF SPOILAGE FUNGI

In response to the increasing demand for natural/mild preserved products, and because of the above studies have shown that the use of sub-optimal concentrations of existing preservatives can reduce lag times and enhance mould growth, further experimentation was carried out in order to identify more natural, alternative antifungal compounds with potential use for control of mould growth on bread products. Plant essential oils and antioxidants were chosen for the study. The initial work was conducted *in vitro* (2% wheat flour agar) and the best compounds validated on bread analogues (*in situ* studies).

4.3.1 Screening for essential oils and antioxidants with antifungal activity on 2% wheat flour agar (*in vitro* studies)

An initial screen on the *in vitro* effect of different antioxidants and essential oils was carried out at a combination of environmental factors that allowed fast growth, i.e., 25°C, 0.95 a_w and pH 6. Experimental conditions were as detailed in section 3.6.1.

Control plates with no preservative were systematically used and analysed for each experiment.

(i) Antioxidants

Table 4.3 shows the level of growth rate inhibition achieved with the different doses of antioxidants. Maximum standard errors of to 0.067mm day^{-1} on triplicate measured growth rates were recorded.

Propyl paraben and BHA were the most effective antioxidants, completely inhibiting the growth of all fungal species at doses of 500 ppm. At a lower concentration (100 ppm), the effect was dependent on the species ($p<005$). *Cladosporium herbarum* was the most sensitive species with 100% inhibition of growth with 100 ppm of both these antioxidants. Overall, BHA was more effective than propyl paraben in reducing growth of the species studied.

On the other hand, BHT and propyl gallate, exhibited poor antifungal activity, with 15-54% inhibition of growth at the maximum dosage tested (1000 ppm). No statistically significant differences between the different doses of BHT were observed ($p>0.05$). Stimulation of growth of the strains of the ochratoxigenic species *P. verrucosum* occurred with 100pm and 500ppm of propyl gallate. Table I-5 in Appendix I shows the analysis of variance of the effect of type of antioxidant, dose and species on lag times and rate of colonisation.

(ii) Essential oils

Tables 4.4 and 4.5 show the effect of 20 different essential oil on lag phases and growth rates respectively. Complete inhibition of all fungi was found with clove, thyme, bay and cinnamon essential oils.

Table 4.3 Percentage of growth rate inhibition of four fungal species growing on 2% wheat flour agar at 25°C, 0.95 a_w and pH 6, in the presence of 100, 500 and 1000 ppm (w/w) of four different antioxidants. Numbers in bold indicate statistically significant values when compared to untreated controls ($p < 0.05$).

GROWTH RATE INHIBITION (%)			
	Dose (ppm;w/w)		
	100	500	1000
Butylated hydroxyanisole (BHA)			
Aspergillus ochraceus	**47**	**100**	**100**
Penicillium verrucosum M450	**83**	**100**	**100**
Penicillium verrucosum M453	**43.7**	**100**	**100**
Penicillium verrucosum PV3	**64**	**100**	**100**
Penicillium corylophilum	**76**	**100**	**100**
Cladosporium herbarum	**100**	**100**	**100**
Butylated Hydroxytoluene (BHT)			
Aspergillus ochraceus	1.7	16	**43**
Penicillium verrucosum M450	9.4	**27.3**	**36.7**
Penicillium verrucosum M453	2.2	12.6	**49.4**
Penicillium verrucosum PV3	-8.7	7.9	**44.7**
Penicillium corylophilum	6.5	**33.6**	**42.3**
Cladosporium herbarum	**23.4**	7.4	**23.4**
Propyl Paraben			
Aspergillus ochraceus	6	**100**	**100**
Penicillium verrucosum M450	**23**	**100**	**100**
Penicillium verrucosum M453	**34.5**	**100**	**100**
Penicillium verrucosum PV3	**21.9**	**100**	**100**
Penicillium corylophilum	**100**	**100**	**100**
Cladosporium herbarum	**100**	**100**	**100**
Propyl Gallate			
Aspergillus ochraceus	6	**16**	**54**
Penicillium verrucosum M450	6.8	6.8	**24.8**
Penicillium verrucosum M453	4.6	17.2	**14.9**
Penicillium verrucosum PV3	**-27.2**	**-32**	**31.5**
Penicillium corylophilum	7.6	**18.3**	**33.6**
Cladosporium herbarum	16	16	**27.2**

Table 4.4 Increase of Lag phases (days) of *Aspergillus*, *Cladosporium* and *Penicillium* isolates on 2% wheat flour agar at 0.95a_w, pH 6 and 25°C, in the presence of 500 ppm (w/w) of different essential oils. Numbers is bold indicate statistically significant values when compared to untreated controls ($p < 0.05$).

	INCREASE IN LAG PHASE (DAYS)				
	Aspergillus Ochraceus	*Cladosporium herbarum*	*Penicillium corylophilum*	*Penicillium verrucosum M450*	*Penicillium verrucosum PV3*
Basil-l*	1.87	**10.40**	1.84	-0.68	1.55
Basil-m*	-0.13	2.49	-0.50	-0.68	-0.22
Bay	**>20**	**>20**	**>20**	**>20**	**>20**
Cinnamon	**>20**	**>20**	**>20**	**>20**	**>20**
Clove	**>20**	**>20**	**>20**	**>20**	**>20**
Eucalyptus	-1.02	0.24	-0.45	-0.91	0.57
Ginger	0.31	0.50	-0.51	-0.20	0.14
Grapefruit	-0.52	1.69	-0.52	0.03	1.47
Lemongrass	4.71	**>20**	3.91	4.00	5.32
Lime	0.20	1.46	0.07	0.78	1.02
Majoram	0.18	2.09	0.00	-0.51	0.65
Nutmeg	1.60	**12.55**	1.57	0.57	-0.27
Orange	0.02	**6.04**	0.50	0.58	0.75
Peppermint	1.72	**7.55**	-0.92	0.48	0.04
Pine-syl	-0.74	0.99	-0.59	-0.17	-0.04
Rosemary	-0.31	2.61	-1.60	-0.72	0.55
Sage	0.80	**5.40**	-0.55	0.33	1.34
Spearmint	3.35	**11.40**	**7.76**	**8.89**	3.26
Sweet fennel	0.93	**8.05**	1.22	0.92	1.32
Thyme	**>20**	**>20**	**>20**	**>20**	**>20**

Table 4.5 Radial growth rate inhibition (%) of *Aspergillus*, *Cladosporium* and *Penicillium* isolates on 2% wheat flour agar at 0.95a_w, pH 6 and 25°C, in the presence of 500 ppm (w/w) of different essential oils. Numbers is bold indicate statistically significant values when compared to untreated controls ($p < 0.05$).

	GROWTH RATE INHIBITION (%)				
	Aspergillus ochraceus	*Cladosporium herbarum*	*Penicillium corylophilum*	*Penicillium verrucosum M450*	*Penicillium verrucosum PV3*
Basil-l*	-16.42	**87.6**	**26.49**	-11.48	-14.77
Basil-m*	-13.51	0.41	12.74	-11.48	**-38.85***
Bay	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Cinnamon	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Clove	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Eucalyptus	-4.73	11.35	8.26	**-25.47**	**-50.39**
Ginger	-6.18	**29.33**	5.71	-3.15	-25.35
Grapefruit	-9.46	5.02	14.67	-12.79	**-75.15**
Lemongrass	**-32.76**	**100.00**	11.80	**5.96**	-10.36
Lime	-6.36	**31.68**	17.67	-11.32	**-53.78**
Majoram	-7.14	-4.11	6.16	-9.23	**-51.73**
Nutmeg	-10.21	**69.85**	7.91	-2.86	**-50.24**
Orange	-19.06	**36.83**	3.25	-14.84	**-34.58**
Peppermint	-19.06	**34.11**	5.62	-21.97	-18.54
Pine-syl	-8.28	12.68	0.96	**-27.37**	-49.41
Rosemary	-7.60	17.84	4.66	-17.46	-53.81
Sage	-19.56	**33.00**	9.24	-17.27	**-71.97**
Spearmint	-21.66	100.00	4.18	**-28.24**	-30.30
Sweet fennel	-21.04	**61.56**	**20.99**	-8.31	**-41.56**
Thyme	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

Growth of the ochratoxigenic species *A. ochraceus* and *P. verrucosum* PV3 was stimulated to some degree in the presence of the other oils tested. However, growth stimulation was not always preceded by a shortening of lag phases. For instance, although growth of *P.verrucosum* (strain M450) was enhanced by the presence of spearmint essential oil, a significant increase of 8 days in the lag phase was observed. Growth of *P. corylophilum* and *C. herbarum* were inhibited to some extent by all the essential oils. A summary of the statistical analysis of the data on the effect of 500 ppm of the different essential oil and species on lag times and rate of colonisation, is shown in Appendix I (Table I-6).

4.3.2 Effect of dose and environmental conditions on the *in vitro* antifungal efficacy of best antioxidants and essential oils.

From the results detailed above, the antioxidants BHA and propyl paraben and essential oils of bay, cinnamon, clove and thyme were chosen as potential alternative compounds for controlling mould spoilage on wheat flour-based products. Further experimentation was focused on the possible application of these compounds to control mould growth on bread under different environments.

For that reason, a narrower set of environmental factors was used in order to better simulate the intrinsic characteristics of bread products. The specific experimental conditions were as detailed in section 3.6.1. Due to the size of the experimental matrix, only two strains of *P.verrucosum* were used (M453 and PV3).

(i) Antioxidants

Figure 4.11 illustrates the effect of increasing concentration of BHA at pH 4.5 and different a_w levels on growth rates of *A.ochraceus*, *P.roquefortii* and *E.repens*. *C.herbarum* was again the species most sensitive to both antioxidants at all a_w and pH levels tested, being completely inhibited with the lowest dose tested (50ppm) at 0.97 a_w over a 30 day incubation period. No further experimentation with antioxidants was carried out with this species.

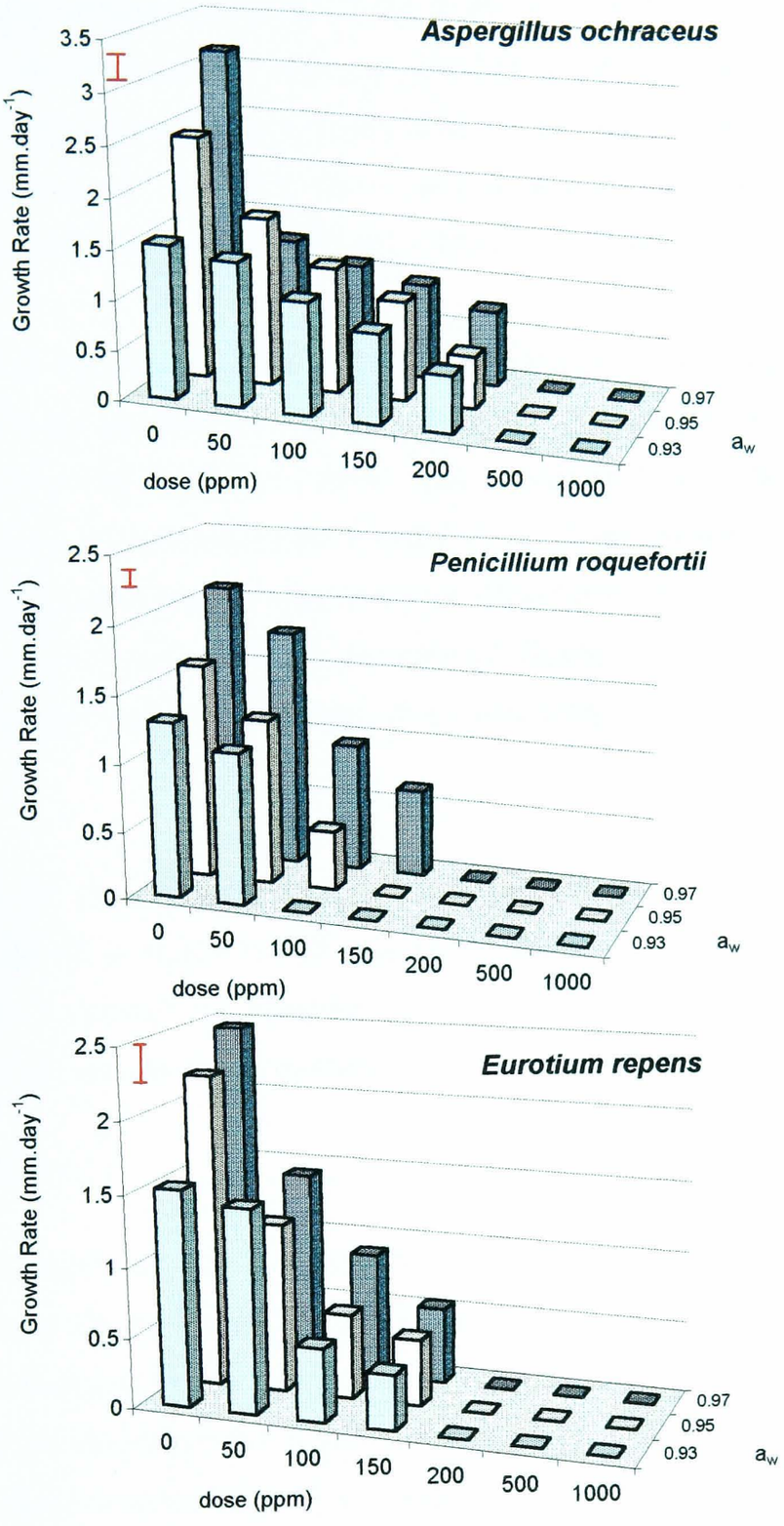


Figure 4.11 Effect of increasing concentrations (ppm) of butylated hydroxyanisole (BHA) on growth rate of *Aspergillus ochraceus*, *Penicillium roquefortii* and *Eurotium repens* growing on 2% wheat flour agar at different water activity levels (a_w), pH 4.5 and 25°C. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

Over all conditions and the same experimental time period, doses of 200 ppm (w/w) were sufficient to control growth of *P.roquefortii*, *P.verrucosum* (both strains) and *E.repens*. On the other hand, *A.ochraceus* grew on media containing 200 ppm of either antioxidants with minimum lag phases and maximum growth rates of 10 days and 0.8 mm day^{-1} respectively. Table 4.6 shows the time at which no growth was observed for any of the species studied at the different a_w levels and dose of antioxidants.

With regard to the type of antioxidant added, generally longer lag phases and lower growth rates were observed with BHA than when propyl paraben was used, particularly at low concentrations. Figure 4.12 shows these effects on growth rates of *P.verrucosum*, *E.repens* and *P.corylophilum* at pH 6 and 0.97 a_w. Some growth enhancement by low doses of antioxidants (50ppm) were observed at the lowest a_w level tested, 0.93. Growth of all species was stimulated in the presence of 50 ppm of propyl paraben at a_w <0.97. Generally, a reduction in growth rate values was preceded by an increase in lag phase duration.

Statistically, all single factors exerted a significant effect on the antifungal activity of both BHA and PP ($p<0.05$). For all species, pH showed little or no effect on lag phases prior to growth although its influence on rate of colonisation was generally significant. The analysis of variance is summarised in Tables I-7a and I-7b in Appendix I.

(i) Essential oils

Figure 4.13 shows the effect of increasing concentrations of cinnamon and clove on growth rates of all species studied at 0.97 a_w and pH 4.5. All four essential oils, bay, clove, cinnamon and thyme were effective at completely inhibiting growth of the test species over all environmental conditions at doses of 1000ppm. *C.herbarum* was again the species most sensitive with 100% inhibition of growth at doses ≥ 100ppm of all four oils. *A.ochraceus* was the only species able to grow in the presence of 500 ppm of all essential oils although only at high a_w (0.95-0.97) and at pH 4.5, with a maximum growth and a minimum lag phase of 0.9 mm day^{-1} and 7 days respectively. At similar conditions, *E.repens* grew with 500 ppm of bay and clove after 10-15 days incubation at a maximum of 0.1 to 0.4 mm day^{-1}.

Table 4.6 Minimum period of time (days) during which no growth was observed for any species (mould-free time) at pH 6, different a_w levels and with increasing concentrations (ppm) of antioxidants butylated hydroxyanisole (BHA) and propyl paraben.

	Mould-free Time (days)		
a_w	**0.97**	**0.95**	**0.93**
control	1.3	1.4	0.8
BHA (ppm)			
50	2.2	1.4	1.2
100	3.6	5.4	1.4
150	6.8	8.2	3.2
200	11.8	9.3	9.9
500	30	30	30
1000	30	30	30
Propyl paraben (ppm)			
50	1.73	2	2.1
100	2.5	2.1	2.5
150	6.4	3.2	5.4
200	12.2	30	30
500	30	30	30
1000	30	30	30

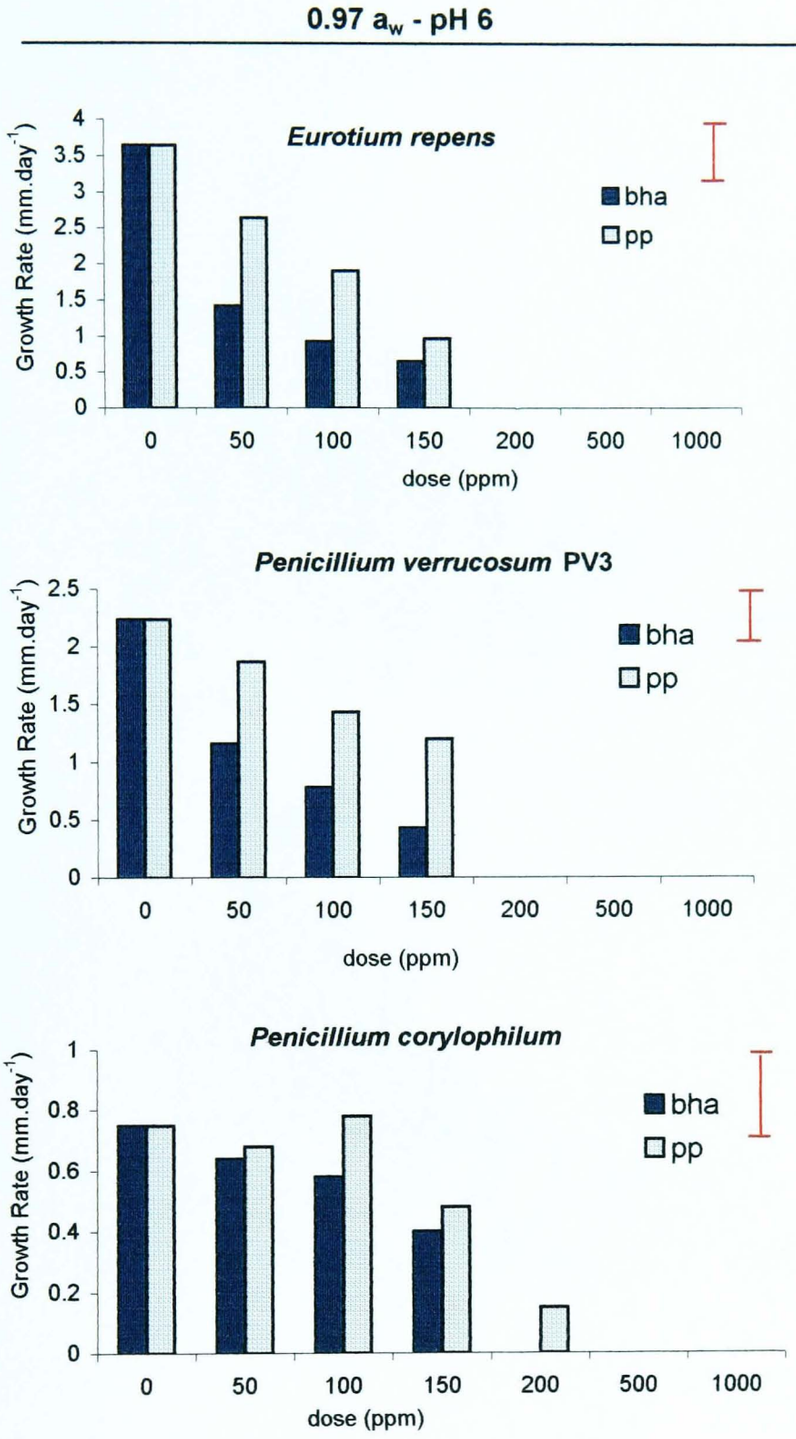


Figure 4.12 Comparative effect of increasing concentrations of butylated hydroxyanisole (BHA) and propyl paraben (PP) on growth rates of *Eurotium repens*, *Penicillium verrucosum* (strain PV3) and *Penicillium corylophilum* growing on 2% wheat flour agar, 0.97 a_w, pH 6 and 25°C. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

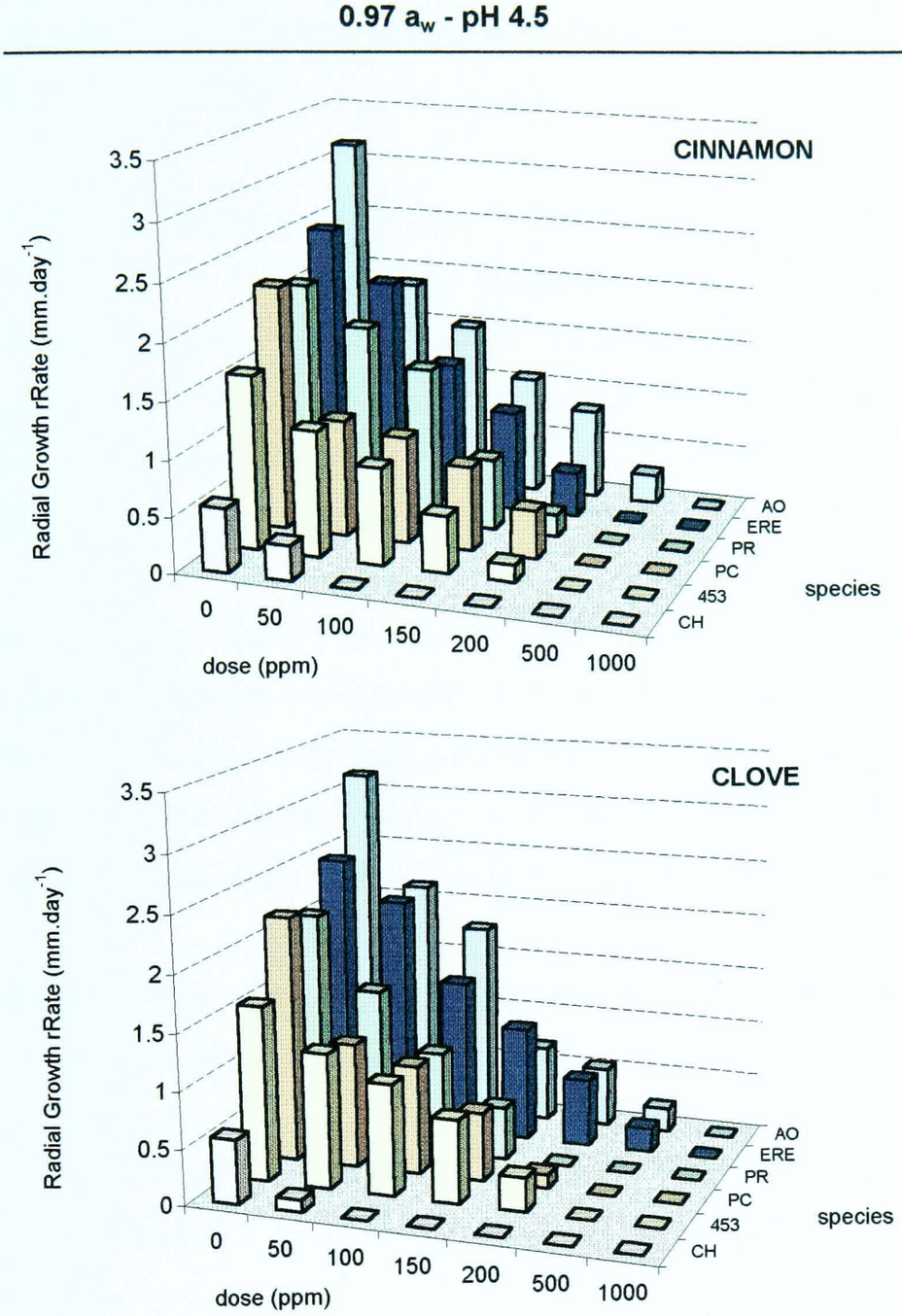


Figure 4.13 Effect of increasing concentrations of cinnamon and clove essential oils on growth rates of *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium roquefortii* (PR), *Penicillium corylophilum* (PC), *Penicillium verrucosum* strain M453 (453) *and Cladosporium herbarum* (CH) growing on 2% wheat flour agar, 0.97 a_w, pH 4.5 and 25°C. All values for each species are significantly different from the controls ($p < 0.05$).

In general terms, the use of all essential oils at concentration of 500 ppm led to a 70-100% growth reduction and an increase in lag phase of 6 days for *A.ochraceus* and up to 20 days for *Eurotium* and *Penicillium* isolates.

Overall, 0.93 a_w and pH 6 was the environmental condition that lead to more effective growth reduction. At these conditions, 200ppm of all oils completely inhibited growth of all species studied. In general terms, oil concentrations needed to completely inhibit mould growth were lower as the a_w of the substrate was reduced. Figure 4.14 shows the effect of a_w on the antifungal activity (% of growth rate inhibition) of thyme essential oil against *A.ochraceus*, *E.repens* and *P.corylophilum* at pH 4.5 and 25°C.

Tables 4.7 and 4.8 illustrate the minimum concentration of each essential oil at which 100% growth inhibition was observed over a period of 30 days for the different species and at the different environmental treatments studied. These data give us information about the Minimum Inhibitory Concentration (MIC) values for each oil which ranged between the values listed in the tables and the immediately lower concentration tested.

No statistically significant differences between the different types of essential oil was observed for *P.roquefortii* and strain M453 of *P.verrucosum*. On the other hand, growth of *A.ochraceus* and *P.verrucosum* (PV3) appeared to be more effectively controlled with cinnamon and bay oil respectively. In Figure 4.15, these differences are illustrated for *A.ochraceus* and *P.verrucosum* strain PV3 at 0.95 a_w and pH 6.

Statistically, from all parameters studied, i.e. a_w, pH, dose and type of oil, only the dose and to a lesser extent a_w, exerted a significant effect on the growth of all species. *A.ochraceus*, *E.repens* and *P.corylophilum* were species significantly affected by all factors and most of the two and three way interactions. Additionally and for all treatments, growth of *P.roquefortii* was significantly slower at pH 6 than at 4.5. No significant differences between the different levels of pH were observed on growth of *P.verrucosum* strains. These differences are summarised in Appendix I (Tables I-8a and I-8b).

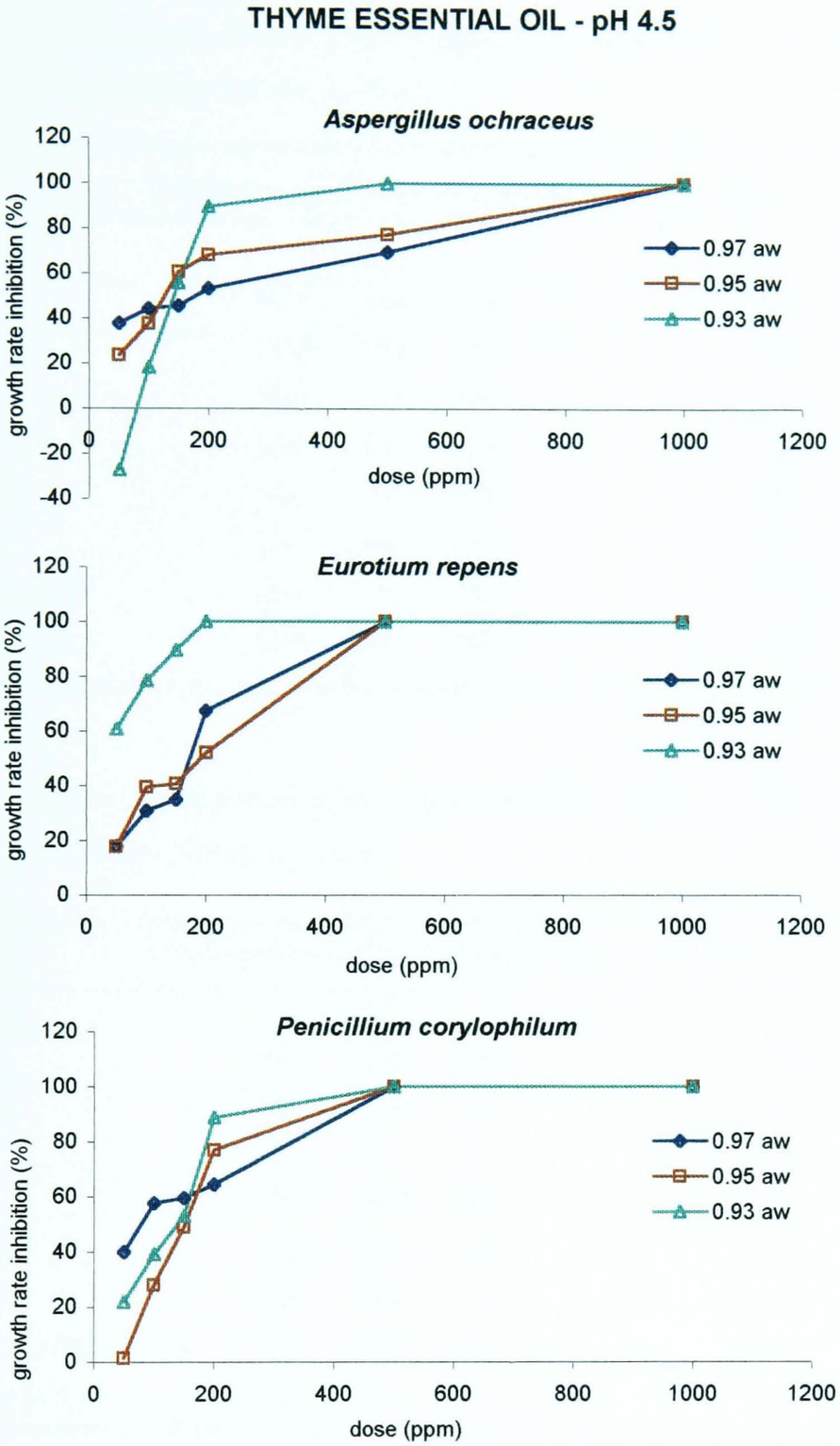


Figure 4.14 Effect of water activity (a_w) on percentage (%) growth inhibition of *Aspergillus ochraceus*, *Eurotium repens* and *Penicillium corylophilum* growing on 2% wheat flour agar, pH 4.5 and 25°C in the presence of increasing concentrations of thyme essential oil.

Table 4.7 Minimum concentration of thyme (ppm; w/w) were 100% of growth rate inhibition was observed at the different pH and a_w levels.

Concentration of THYME essential oil (ppm; w/w)						
pH		**4.5**			**6**	
a_w	**0.97**	**0.95**	**0.93**	**0.97**	**0.95**	**0.93**
A.ochraceus	1000	1000	500	500	500	200
C.herbarum	100	100	100	100	100	100
E.repens	500	500	200	500	500	150
P.corylophilum	500	500	500	200	200	200
P.roquefortii	200	200	150	200	200	100
P.verrucosum M453	200	200	200	200	200	150
P.verrucosum PV3	200	500	200	1000	200	150

Table 4.8 Minimum concentration of bay (ppm; w/w) were 100% of growth rate inhibition was observed at the different pH and a_w levels.

Concentration of BAY essential oil (ppm; w/w)						
pH		**4.5**			**6**	
a_w	**0.97**	**0.95**	**0.93**	**0.97**	**0.95**	**0.93**
A.ochraceus	1000	1000	500	1000	500	200
C.herbarum	100	100	50	100	100	50
E.repens	1000	1000	200	500	500	200
P.corylophilum	1000	500	500	200	500	200
P.roquefortii	500	500	150	200	200	150
P.verrucosum M453	500	500	200	500	200	200
P.verrucosum PV3	500	200	200	500	200	200

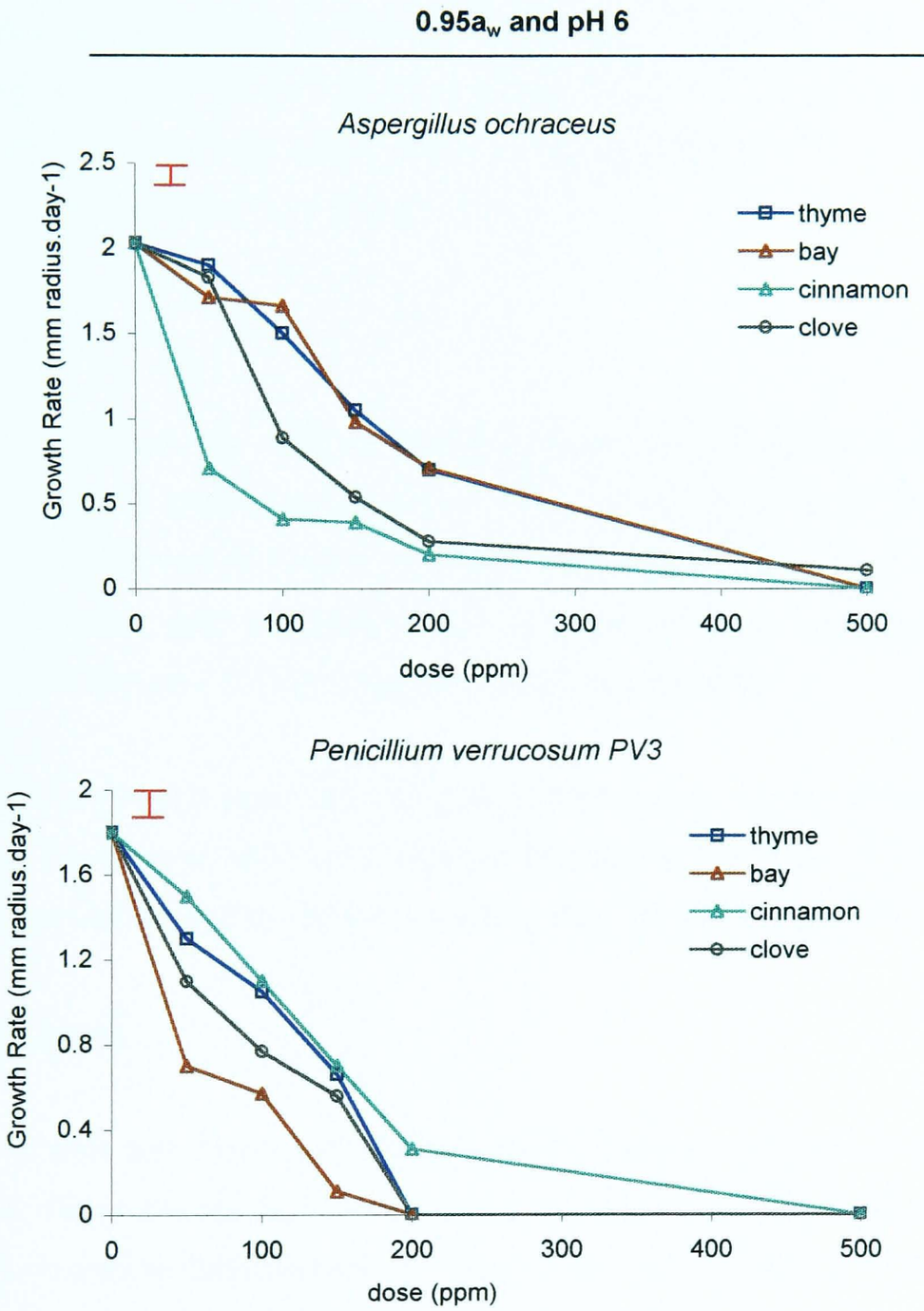


Figure 4.15 Comparative effect of increasing concentrations (ppm) of thyme, bay, cinnamon and clove oils on growth rates of *Aspergillus ochraceus* and *Penicillium verrucosum* (strain PV3) growing on 2% wheat flour agar, 0.95 a_w, pH 6 and 25°C. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

4.3.3 Efficacy of best antioxidants and essential oils on bread analogues

The efficacy of selected antioxidants and essential oils was determined on bread analogues in order to assess the effect of substrate on their proven antifungal activity. The antioxidants BHA and PP and essential oils of bay, clove, cinnamon and bay were incorporated into the bread dough at final concentrations of 100, 300, 500 and 1000 ppm (w/w). The study was carried out at 0.97a_w.

(i) Antioxidants

Figure 4.16 depicts the effect of increasing doses of BHA and propyl paraben on growth rates of all species at pH 4.5 and 0.97 a_w. No effect on lag phases and growth rate of any species was observed with doses of antioxidants ≤ 300ppm. *Penicillium* and *Eurotium* isolates were only significantly inhibited with the highest dose of both antioxidants although < 30% inhibition of growth was recorded.

Growth of *A.ochraceus* appeared to be more effectively controlled with propyl paraben than BHA. For instance, while no inhibitory effect was observed with 500 ppm of BHA, the same concentration of propyl paraben led to 36% reduction in growth.

(ii) Essential oils

Similar to what was found with antioxidants, the addition of essential oils of clove, cinnamon, thyme and bay in bread analogues showed little effect in controlling mould growth compared to that observed on WFA. Statistically significant effects were only observed for *E.repens* at oil concentrations ≥ 500ppm with growth reductions between 27-52%. Figure 4.17 illustrates the effect of increasing concentrations of essential oils on growth of *A.ochraceus, E.repens* and *P.corylophilum* at pH 6 and 0.97a_w.

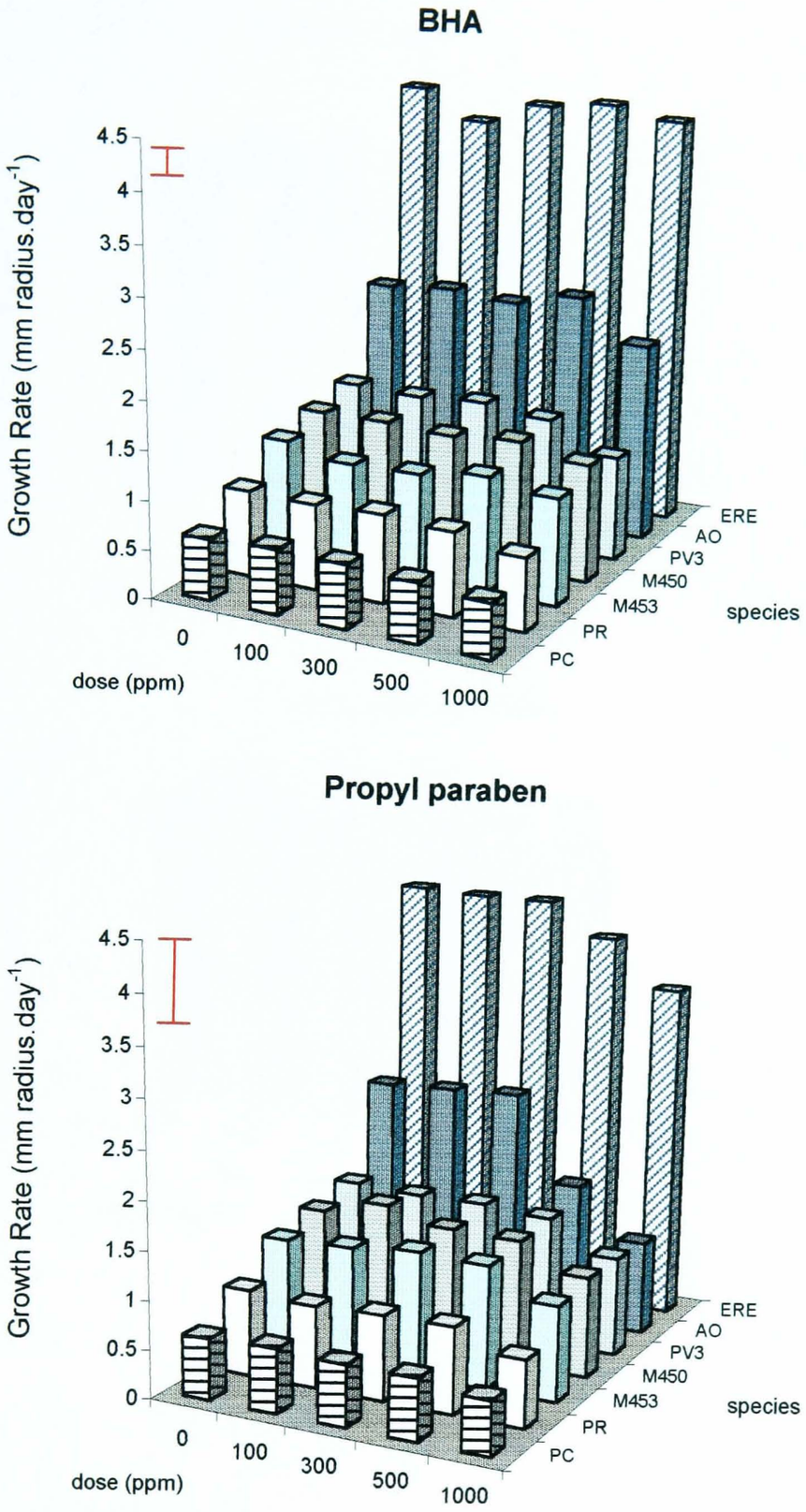


Figure 4.16 Effect of increasing concentrations of Butylated hydroxyanisole (BHA) and propyl paraben (PP) on growth rates of *Eurotium repens* (ERE), *Aspergillus ochraceus* (AO), *Penicillium verrucosum* (strains M450, M453 an PV3), *Penicillium roquefortii* (PR) and *Penicillium corylophilum* (PC) on bread analogues at 0.97 a_w, pH 4.5 and 25°C. Bars indicate Least Significant Differences (LSD) at $p<0.05$.

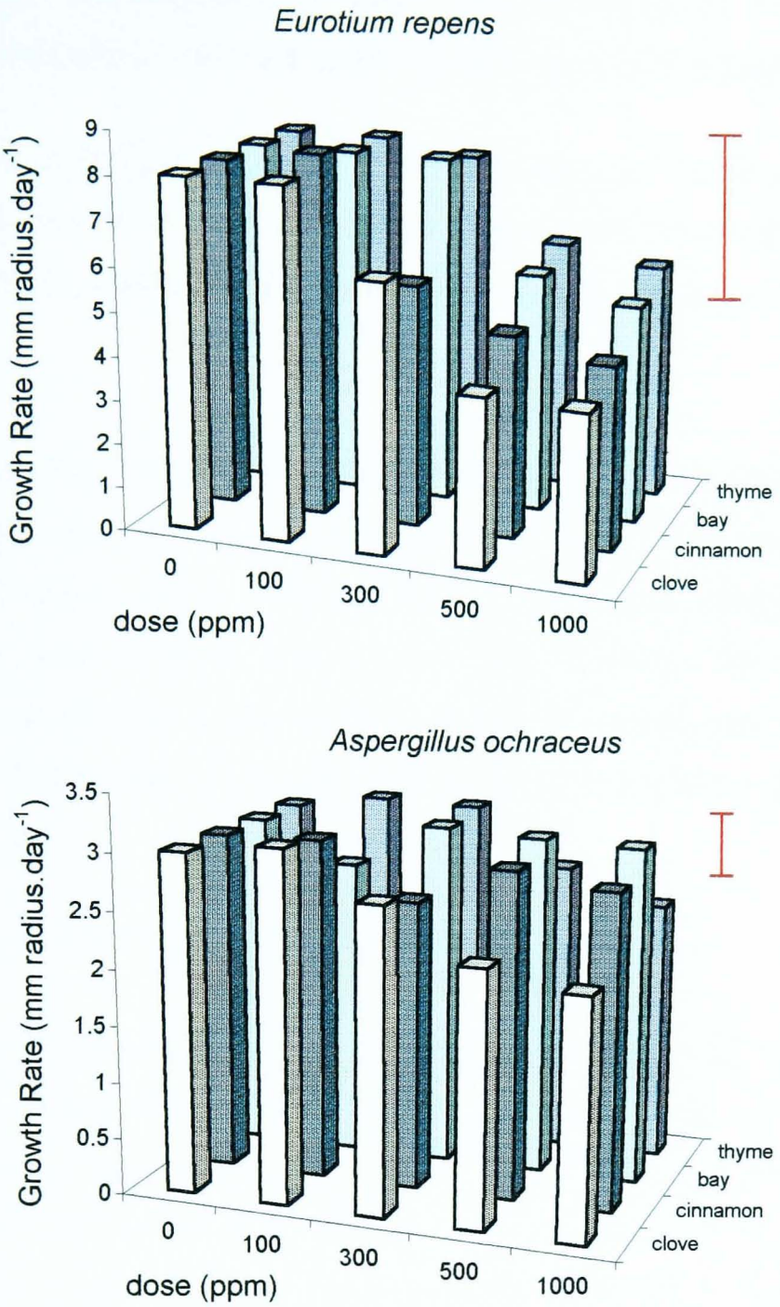


Figure 4.17 Comparative effect of increasing concentrations of thyme, bay, cinnamon and clove essential oils on growth rates of *Eurotium repens* and *Aspergillus ochraceus* growing on bread analogues at 0.97 a_w, pH 6 and 25°C. Bars indicate Least Significant Differences (LSD) at $p<0.05$.

4.4 EFFECT OF PRESERVATIVES AND ENVIRONMENT ON OCHRATOXIN A (OTA) PRODUCTION BY *A.OCHRACEUS* AND *P.VERRUCOSUM*

As detailed in Chapter 1, a very important aspect of mould spoilage of foods is the possible formation of toxic metabolites (mycotoxins) by the growing fungi. In wheat-based products, formation of ochratoxins by *Aspergillus* and *Penicillium* spp is of great concern.

For that reason, the potential for ochratoxin A (OTA) production by cultures of *A.ochraceus* and *P.verrucosum* was determined at 25°C both on wheat flour agar and on bread analogues, under different environments and preservation factors. Toxin production was screened over a period of 54 days. The effect of the existing preservatives, potassium sorbate and calcium propionate, and the potential alternative compounds, NHA, propyl paraben and essential oils of thyme, clove, cinnamon and bay was studied.

4.4.1 Ochratoxin A production on 2% wheat flour agar.

In the initial screen on 2% WFA in the absence of preservatives (see section 3.7.1), no OTA was produced by any of the species studied over the experimental period. Subsequently, it was decided to carry out the same temporal study in the presence of preservatives in order to establish possible stress-effects on OTA production by these species. One concentration of all preservatives (150 ppm) was used for comparison. Potassium sorbate was also added at an additional concentration of 300ppm for comparison with *in vitro* results.

Similarly, no OTA production was found in treatments containing 150 ppm of BHA, PP, or thyme, clove, cinnamon and bay essential oils, at any time or a_w level tested. Interestingly, OTA was detected in media containing sub-optimal concentrations of potassium sorbate (150 and 300 ppm). Production was only observed in cultures of *P.verrucosum* strain PV3 after 28 days incubation at all a_w levels (Figure 4.18) and by *P.verrucosum* strain M453 after 54 days.

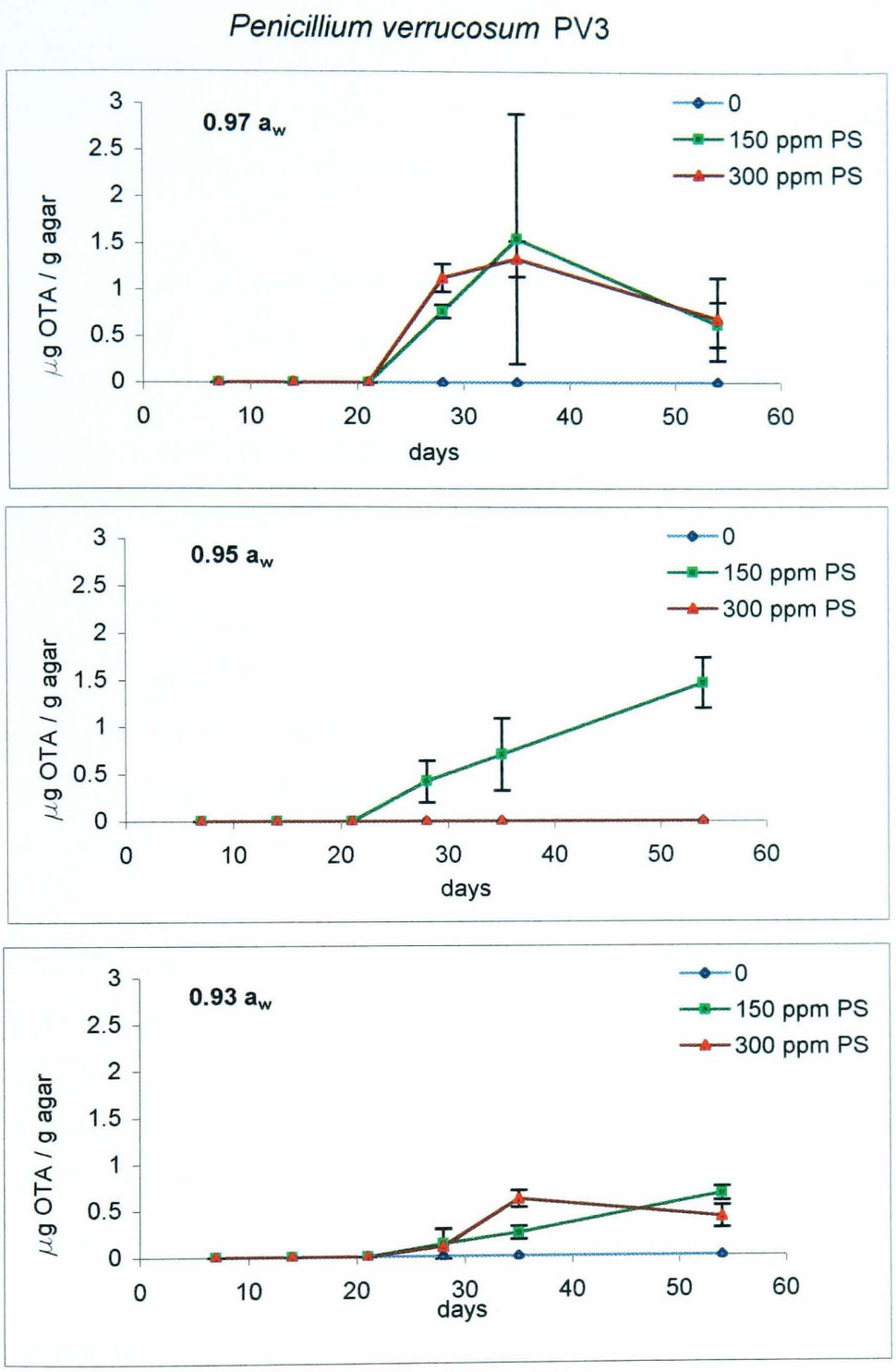


Figure 4.18 OTA contents ($\mu g\ g^{-1}$) produced by *Penicillium verrucosum* strain PV3 at pH 6, and three water activity levels (a_w) in the presence of 150 and 300 ppm of potassium sorbate (PS). Points are the mean of three replicates and bars indicate ± standard deviations.

Generally, OTA concentrations were significantly higher as time of incubation and a_w level increased ($p<0.05$) with a maximum production of 1.5 $\mu g\ g^{-1}$. High variability on OTA production between replicates was recorded especially at the highest a_w level tested, 0.97 (standard deviations of up to 1.33 $\mu g\ OTA.g^{-1}$).

The presence of potassium sorbate in the substrate exerted a significant effect ($p<0.001$) on OTA production, although no significant differences ($p>0.05$) were observed between plates containing 150 and 300ppm of the preservative. Table I-9 in Appendix I shows the analysis of variance on the effect of a_w, day and dose of potassium sorbate on OTA production by cultures of strain PV3 of *P.verrucosum.*

A.ochraceus and *P.verrucosum* strain M450 produced no detectable OTA when grown on 2% WFA for up to 54 days. *A.ochraceus* however, produced a consistent peak at retention times of ca. 4.5 minutes which showed similar variations in relation to a_w, concentration of preservative and time of incubation to those observed for ochratoxin A (retention times of 6 minutes).

In an attempt to identify this peak, samples were analysed again co-injected with standards of ochratoxin B (OTB) which can also be produced by laboratory cultures (Moss, 1996) and have a slightly shorter retention time than OTA under the same detection conditions. However, chromatograms resulting from the co-injected samples in comparison with those for OTB standards, rejected the hypothesis that OTB was the unknown peak.

4.4.2 Ochratoxin A production on bread analogues

(i) Temporal effect of environmental conditions on toxin production

Figures 4.19 and 4.20 illustrate the production of OTA over time by cultures of *P.verrucosum* grown on bread analogues and at different a_w and pH levels. On bread analogues and over a 35 day period, *A.ochraceus* did not produce any OTA at any time, a_w or pH studied.

Penicillium verrucosum at pH 4.5

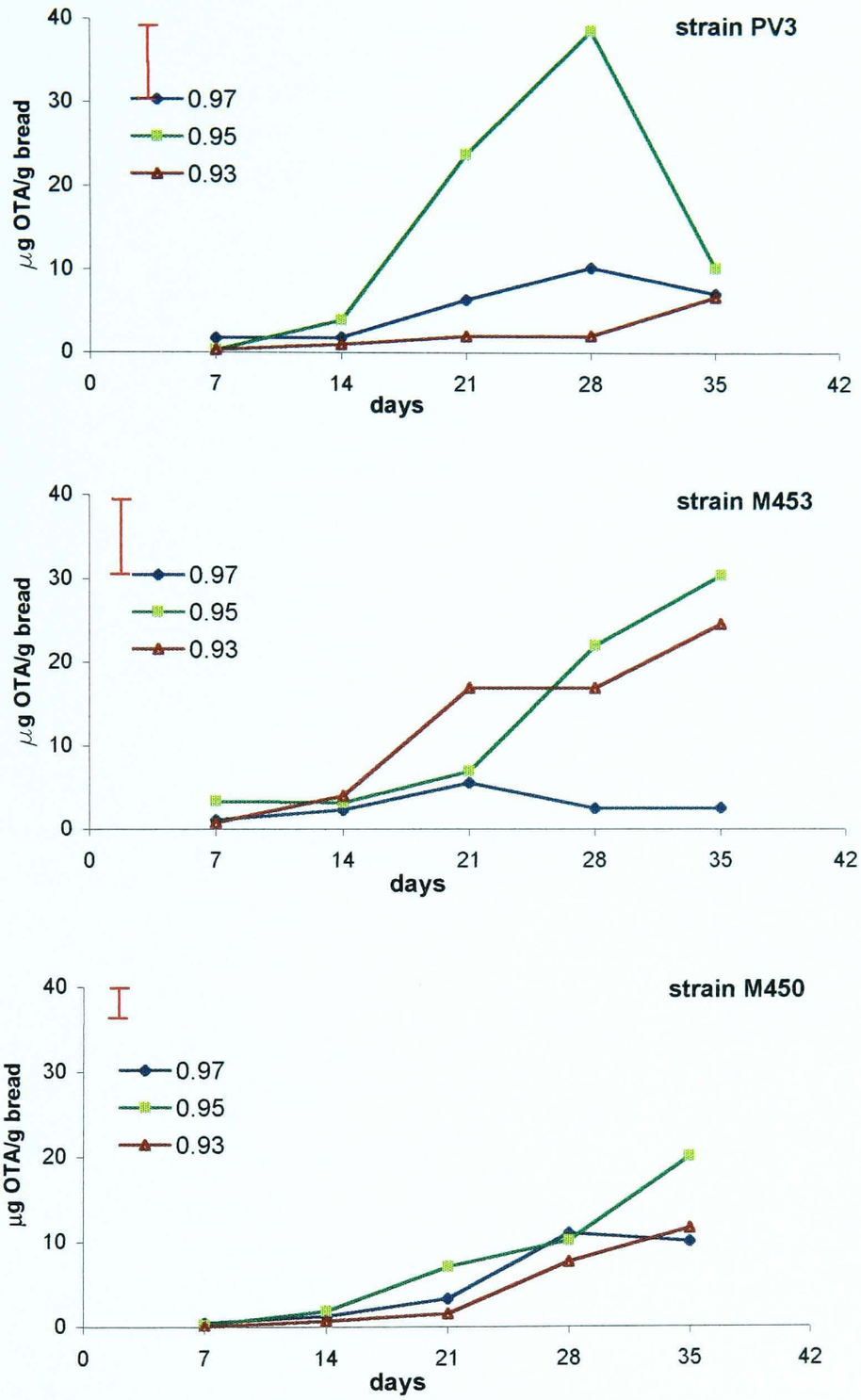


Figure 4.19 Temporal ochratoxin A production (µg g^{-1}) by cultures of different strains of *Penicillium verrucosum* (strains M450, M453 and PV3) growing on bread analogues at pH 4.5 and different water activity levels (a_w) (means of three replicates per treatment). Bars represent Least Significant Differences (LSD) at $p < 0.05$.

Penicillium verrucosum at pH 6

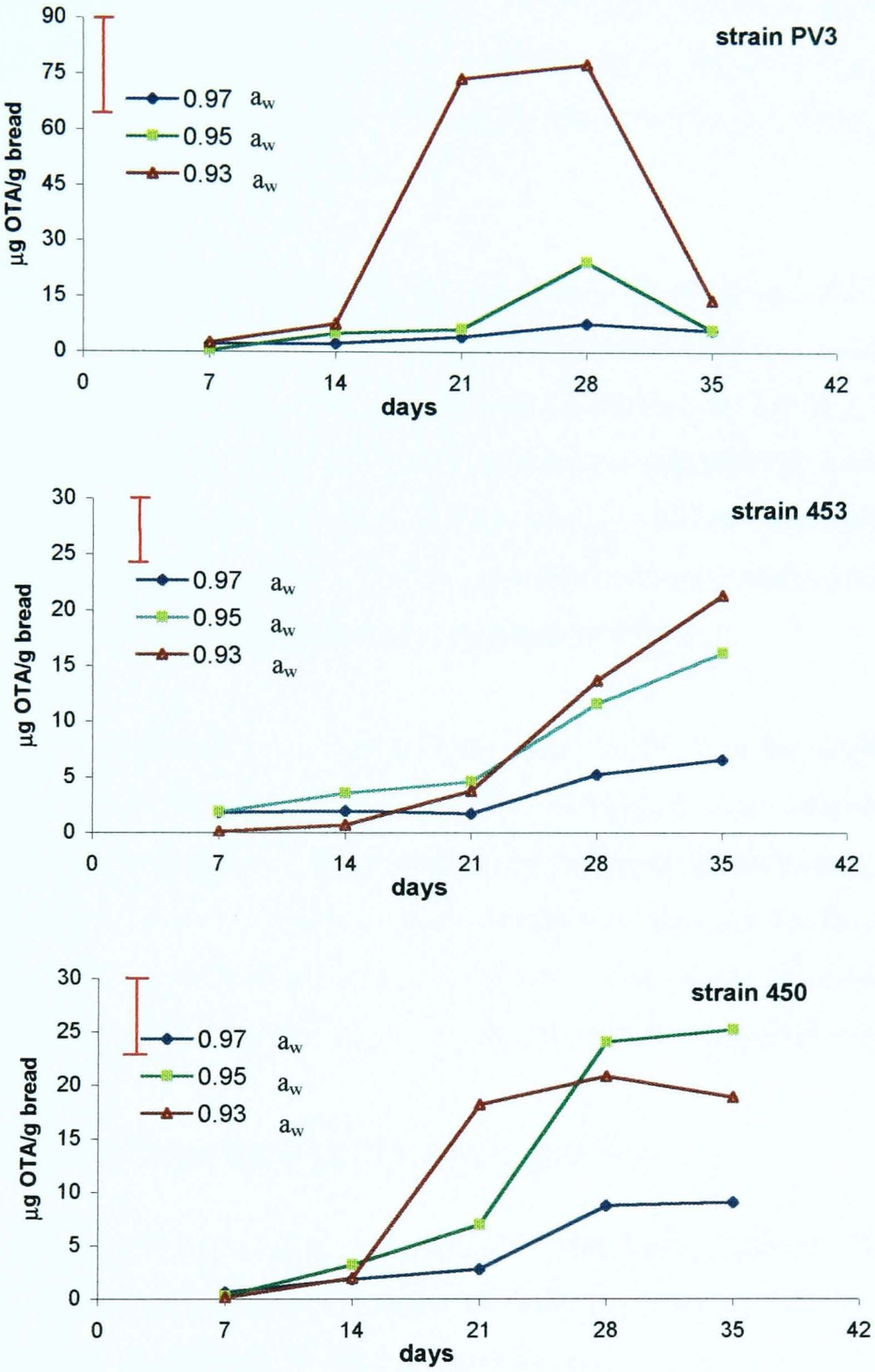


Figure 4.20 Temporal ochratoxin A production (µg g^{-1}) by cultures of different strains of *Penicillium verrucosum* (strains M450, M453 and PV3) growing on bread analogues at pH 6 and different a_w (mean of three replicates per treatment). Bars represent Least Significant Differences (LSD) at $p < 0.05$.

All strains of *P.verrucosum* produced up to 3.25 $\mu g\ g^{-1}$ after only 7 days incubation with a maximum production at 28 or 35 days depending on species and treatment. The greatest OTA production was observed at pH 6 and at the lowest a_w levels, 0.95 and 0.93. For instance, *P.verrucosum* strain PV3 after 28 days of incubation growing at pH 6 and 0.93 a_w produced up to 75 ppm OTA while only 7.5 ppm at 0.97 a_w at the same pH level was detected (see Figure 3.20).

The a_w treatment significantly affected OTA production by all strains of *P.verrucosum* from the first day of analysis (7 days incubation). The effect of pH on toxin production appeared to be dependent upon strain and time of incubation. In fact, while OTA concentrations produced by strain PV3 of *P.verrucosum* significantly varied with pH ($p<0.001$) from day 7, strains M450 and M453 were only affected ($p<0.05$) between 14 and 28 days of incubation. At pH 6, 0.93 a_w appeared to be the optimum a_w for toxin production while at pH 4.5, production was enhanced at 0.95 a_w.

In all cases, OTA production was significantly lower ($p<0.05$) at the highest a_w level tested (0.97). Again, high variability in OTA production was observed between treatments and within replicates. Overall treatments and times of incubation, a statistical difference ($p<0.05$) in the potential for OTA production between the three strains of *P.verrucosum* was observed. Table I-10 in Appendix I summarises the results from the analysis of variance on the effect of strain, a_w, pH and day on ochratoxin A production.

(ii) Effect of type and concentration of existing preservatives

Due to the high variability of toxin production observed, controls of all strains with no preservative, were made and analysed again for every set of a_w x pH treatment studied. As expected, OTA contents at 28 days of incubation on repeated control plates were significantly different to those detected in the temporal study ($p<0.001$ for strains M450 and M453 and $p<0.05$ for strain PV3). However, the differences between treatments followed similar patterns as previously detailed.

No toxin was detected at pH 4.5 in media containing 3000 ppm of potassium sorbate or calcium propionate (Figure 4.21). OTA levels up to 7.5 ppm were detected when concentrations were reduced to a sub-optimal level of 300 ppm at pH 4.5. At pH 6, detectable levels of OTA were produced by all strains of *P.verrucosum* for all treatments studied (Figure 4.22). Generally, lower OTA concentrations were observed when either preservative was added into the substrate when compared to the untreated controls ($p<0.05$).

Over all a_w levels tested, a maximum of 3 $\mu g\ g^{-1}$ OTA were recorded in the presence of 3000 ppm of both salts at pH 6. Similar to that found *in vitro*, stimulation of OTA production in cultures of *P.verrucosum* M453 growing at pH 6 in media containing 300 ppm of both preservatives was observed. For instance, a significant increase ($p<0.05$) from 15 to more than 40 $\mu g\ g^{-1}$ at 0.93 a_w, pH 6 and with 300 ppm of calcium propionate was recorded (see Figure 4.22).

Concentration of preservative, a_w and pH were the parameters that exerted significant effects on OTA production ($p<0.05$) of all strains with both organic acids (see Appendix I; Table I-10). No statistically significant differences on toxin production ($p>0.05$) between potassium sorbate and calcium propionate were observed for strain PV3 of *P. verrucosum* (Appendix I; Tables I-11a and I-11b).

(iii) Effect of antioxidants and essential oils

Table 4.9 shows the μg of OTA produced per gram of bread analogue by all three strains of *Penicillium verrucosum* at 0.97 a_w and pH 6 in the presence of 1000ppm of antioxidants and essential oils comparing it to the untreated samples. Production of OTA by strains M450 and PV3 of *P.verrucosum* was generally reduced by the addition of all antioxidants and essential oils.

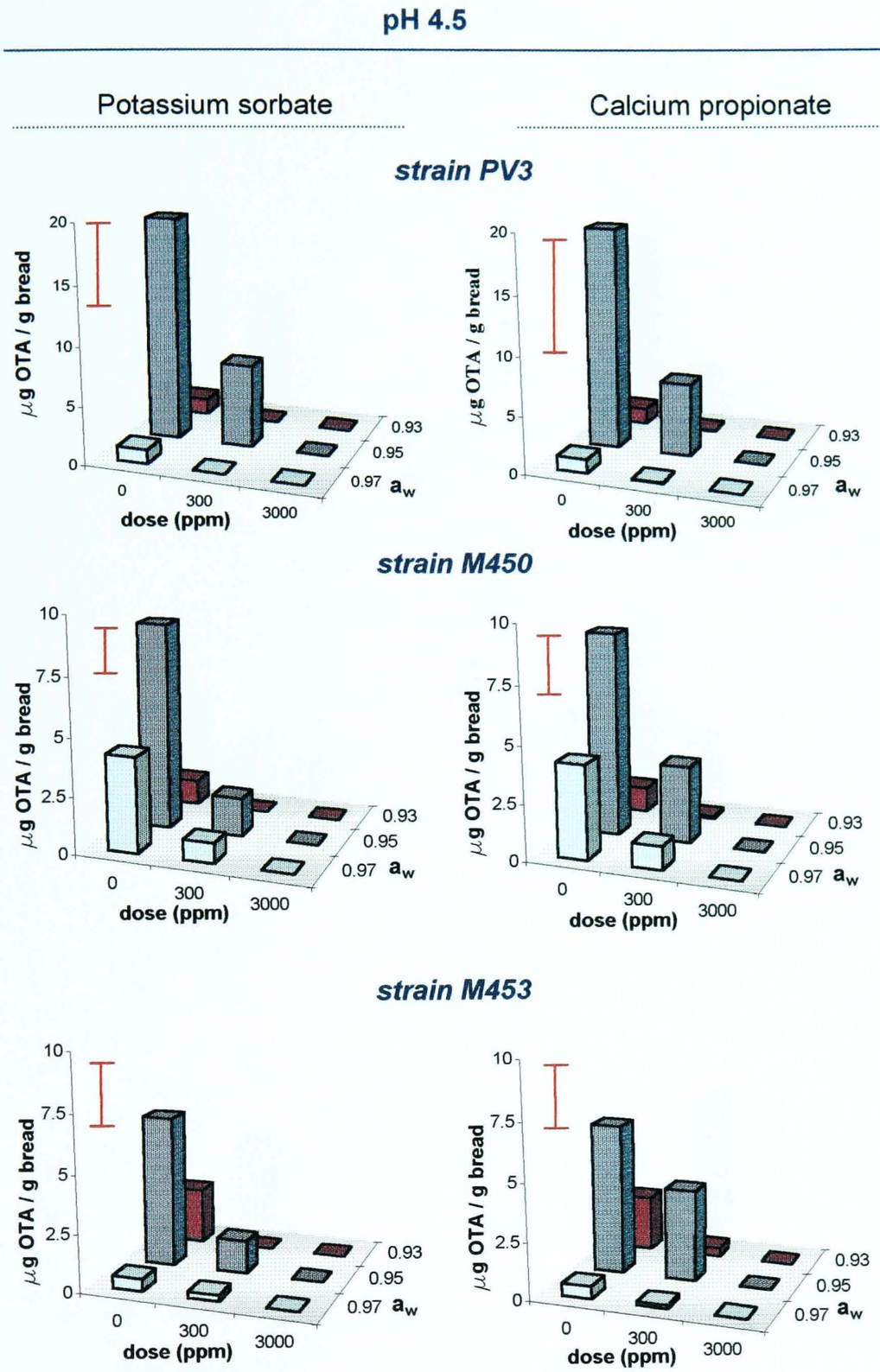


Figure 4.21 Ochratoxin A production (μg g^{-1}) by cultures of *Penicillium verrucosum* (strains PV3, M450 and M453) after 28 days incubation at 25°C on bread analogue at pH 4.5, different water activity (a_w) levels and in the presence of different concentrations of potassium sorbate and calcium propionate. Bars represent Least Significant Differences (LSD) at $p < 0.05$.

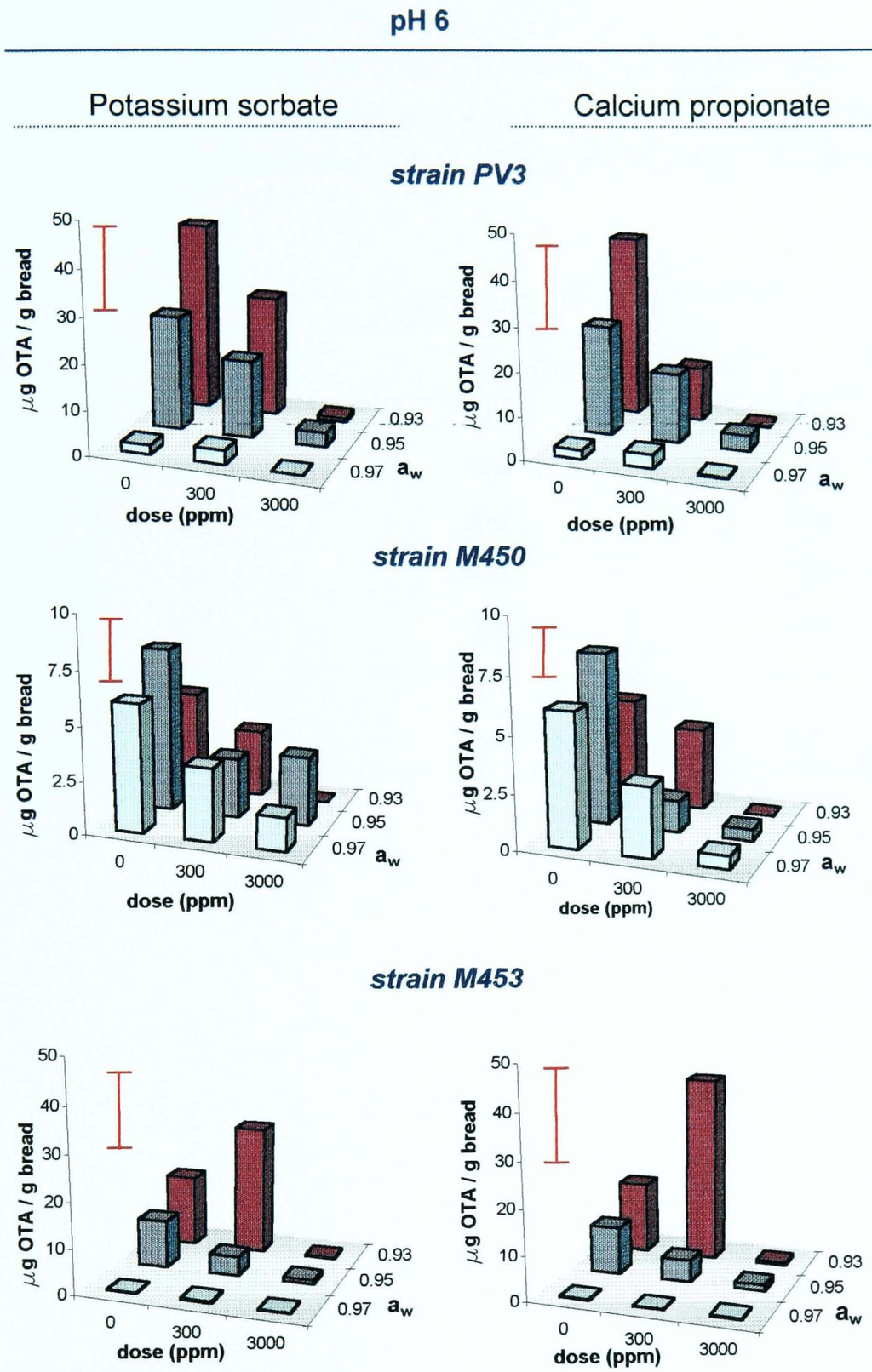


Figure 4.22 OTA production (μg g^{-1}) by cultures of *Penicillium verrucosum* (strains PV3, M450 and M453) after 28 days incubation at 25°C on bread analogue at pH 6, different water activity (a_w) levels and in the presence of different concentrations of potassium sorbate and calcium propionate. Bars represent Least Significant Differences (LSD) at $p < 0.05$.

Table 4.9 OTA contents (μg g^{-1}) of *Penicillium verrucosum* cultures growing on bread analogues, at 0.97 a_w and pH 6 and in the presence of 1000 ppm of antioxidants and essential oils (values are mean of three replicates). Numbers in bold indicate values significantly different from controls ($p < 0.05$).

	μg OTA g $bread^{-1}$		
Strain	**M453**	**M450**	**PV3**
Control	4.51	6.00	7.00
BHA	7.06	**1.05**	4.19
PP	5.24	**0.63**	**1.74**
CLOVE	3.52	**2.99**	7.00
THYME	7.37	**0.60**	**1.53**
CINNAMON	1.45	**0.89**	2.23
BAY	3.06	**0.26**	6.28

On the other hand, strain M453 produced higher amounts of OTA compared to the untreated samples ($p>0.05$) when the antioxidants BHA and PP and the essential oil thyme were added to the substrate. Although differences on toxin production between untreated and treated samples were significant ($p<0.001$), no statistical variance between type of preservative was observed.

4.5 EFFECT OF ENVIRONMENT AND SUB-OPTIMAL CONCENTRATION OF POTASSIUM SORBATE ON CARBON SOURCES UTILISATION PROFILES AND NICHE OVERLAP BETWEEN BREAD SPOILAGE FUNGI

The onset and diversity of fungal spoilage on bread, as in any food product, is dependent upon several factors. Environmental factors such as a_w and temperature has been proven to affect the competitiveness and interactions between species of field spoilage fungi within an ecosystem (Marin *et al.*, 1998; Lee and Magan, 1999). However, species interactions on post-processed food products and the effect of other important factors such as pH or presence of sub-optimal concentrations of preservatives, have been surprisingly neglected.

It has already been proven that sub-optimal concentrations of potassium sorbate can enhance the rate of colonisation of some bread spoilage moulds (see sections 4.1 and 4.2). Enhancement of growth of some species and not others can lead to changes in the dynamics of the spoilage bioburden. Moreover, changes in the fungal community interactions may impact growth and mycotoxin production by ochratoxigenic species such as *A.ochraceus* and *P.verrucosum* with the subsequent impact on consumer safety.

The amount of different carbon sources that a species is able to catabolize in a certain environment, compared to those utilised by its surrounding species, gives us information about its degree of nutritional dominance. A total of 32 different carbon sources (CS) normally present in wheat flour-based products were used to assess the nutritional diversity and interaction between common bread spoilage moulds in different environments (see section 3.8.1).

4.5.1 Effects on number of carbon sources utilised (Niche Size)

(i) Effect of environmental conditions

Table 4.10 shows the minimal a_w levels for CS utilisation for all species and treatments and Figure 4.23 depicts the effect of a_w and pH on niche sizes of *P.corylophilum, C.herbarum, P.verrucosum* strain M450 and *P.roquefortii*, at 25°C and the two pH levels studied.

In general, for all treatments studied (T x pH x dose combinations), the number of carbon sources utilised by a specific species significantly decreased ($p < 0.05$) as the a_w of the medium was reduced. At 0.97 a_w and 25°C all species utilised from 15 to 25 CS which represent from 50 to 80% of the total CS available. Mycotoxigenic strains of *P.verrucosum* (M450, M453 and PV3) generally had bigger niche sizes, utilising up to 13 CS (40% of total available) at the lowest a_w level tested, 0.90. For all conditions, *C.herbarum* was unable to used any carbon source at a_w <0.95.

Figure 4.24 illustrates the effect of temperature on niche sizes of all species at 0.97a_w and at pH 5 and 6.5. Temperature effects on niche size appeared to be species dependent. The effect of temperature over all treatments on total CS utilisation for *E.repens, A.ochraceus* was statistically significant ($p < 0.05$). *E.repens* was most sensitive to a decrease in temperature with a general niche size reductions of 70-100%.

Regarding pH, bigger niche sizes at pH 5 than 6.5 were generally observed (see Figure 4.23). This difference was, however, only statistically significant for *E.repens, P.corylophilum* and strain M453 of *P.verrucosum.* The effect of pH on niche sizes became significant ($p<0.05$) for all species when potassium sorbate was added to the media (see Appendix I; Table I-13).

Table 4.10 Minimum a_w level for CS utilisation for all species studied at the different pH and temperature levels and in the presence or absence of potassium sorbate (PS).

Minimum a_w (Niche Size)				
pH	**5**		**6.5**	
PS concentration (ppm)	0	300	0	300
25 °C				
Aspergillus ochraceus	0.90 (9)	0.93 (1)	0.90 (6)	0.90 (3)
Cladosporium herbarum	0.95 (12)	0.95 (1)	0.95 (11)	0.95 (2)
Eurotium repens	0.90 (3)	0.97 (12)	0.90 (1)	0.90 (7)
Penicillium corylophilum	0.90 (5)	0.97 (3)	0.90 (2)	0.93 (1)
Penicillium roquefortii	0.93 (9)	0.95 (1)	0.93 (2)	0.95 (5)
Penicillium verrucosum PV3	0.90 (10)	0.93 (2)	0.90 (13)	0.90 (10)
Penicillium verrucosum M450	0.90 (10)	0.95 (1)	0.90 (2)	0.90 (2)
Penicillium verrucosum M453	0.90 (12)	0.95 (1)	0.90 (5)	0.90 (4)
15°C				
Aspergillus ochraceus	0.90 (1)	0.95 (2)	0.93 (6)	0.93 (1)
Cladosporium herbarum	0.95 (12)	0.95 (1)	0.95 (11)	0.95 (15)
Eurotium repens	0.93 (2)	0.97 (6)	0.93 (2)	0.93 (9)
Penicillium corylophilum	0.90 (6)	0.97 (3)	0.93 (2)	0.90 (1)
Penicillium roquefortii	0.95 (8)	0.95 (6)	0.93 (1)	0.93 (1)
Penicillium verrucosum PV3	0.90 (11)	0.95 (4)	0.90 (9)	0.90 (8)
Penicillium verrucosum M450	0.90 (13)	0.95 (2)	0.90 (4)	0.90 (8)
Penicillium verrucosum M453	0.90 (15)	0.95 (2)	0.90 (13)	0.90 (8)

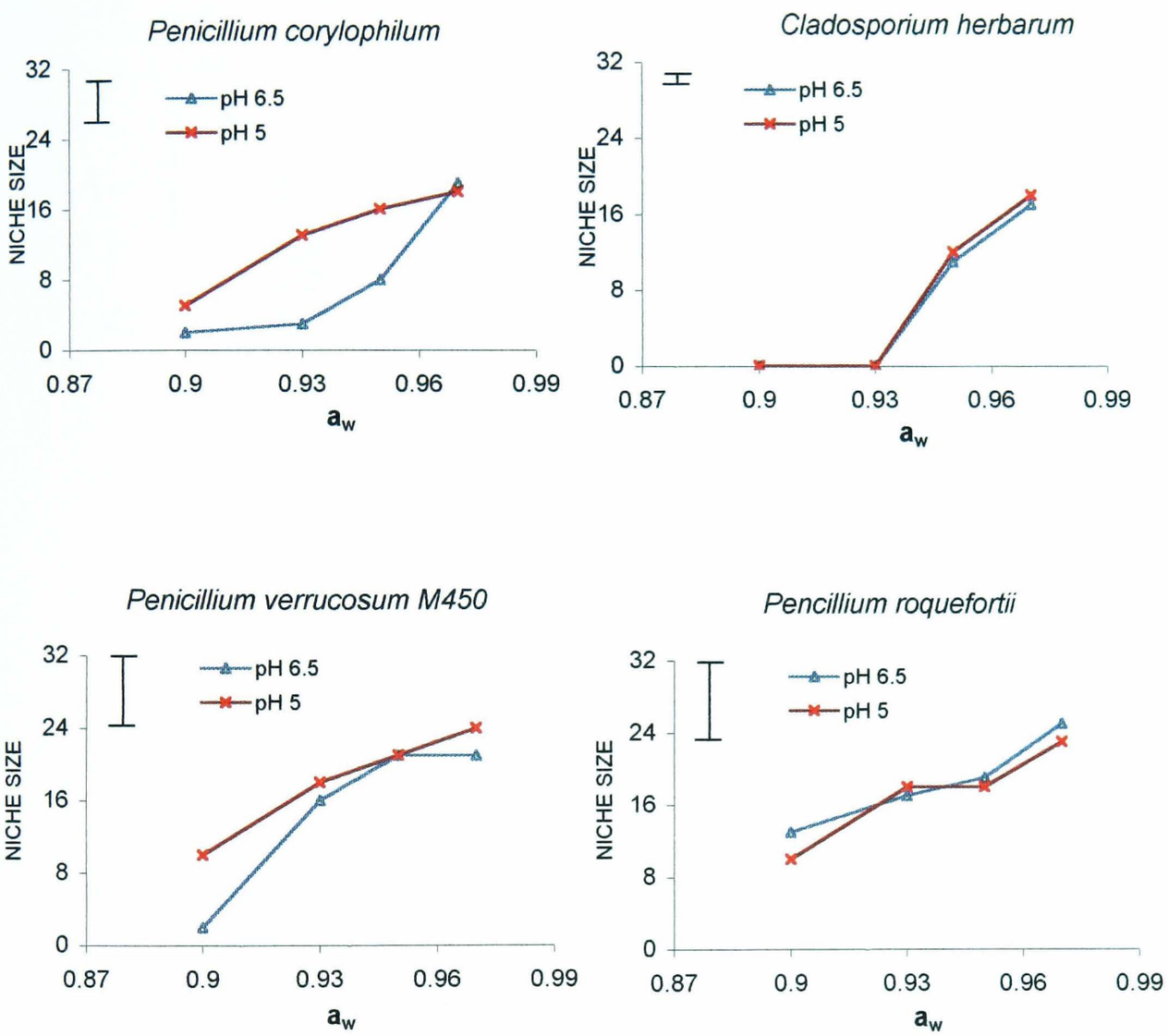


Figure 4.23 Effect of water activity (a_w) on the niche size of *Penicillium corylophilum, Cladosporium herbarum, Penicillium roquefortii* and strain M450 of *Penicillium verrucosum* at 25°C and two different pH levels, 5 and 6.5. Bars indicate Least significant Differences at $p < 0.05$.

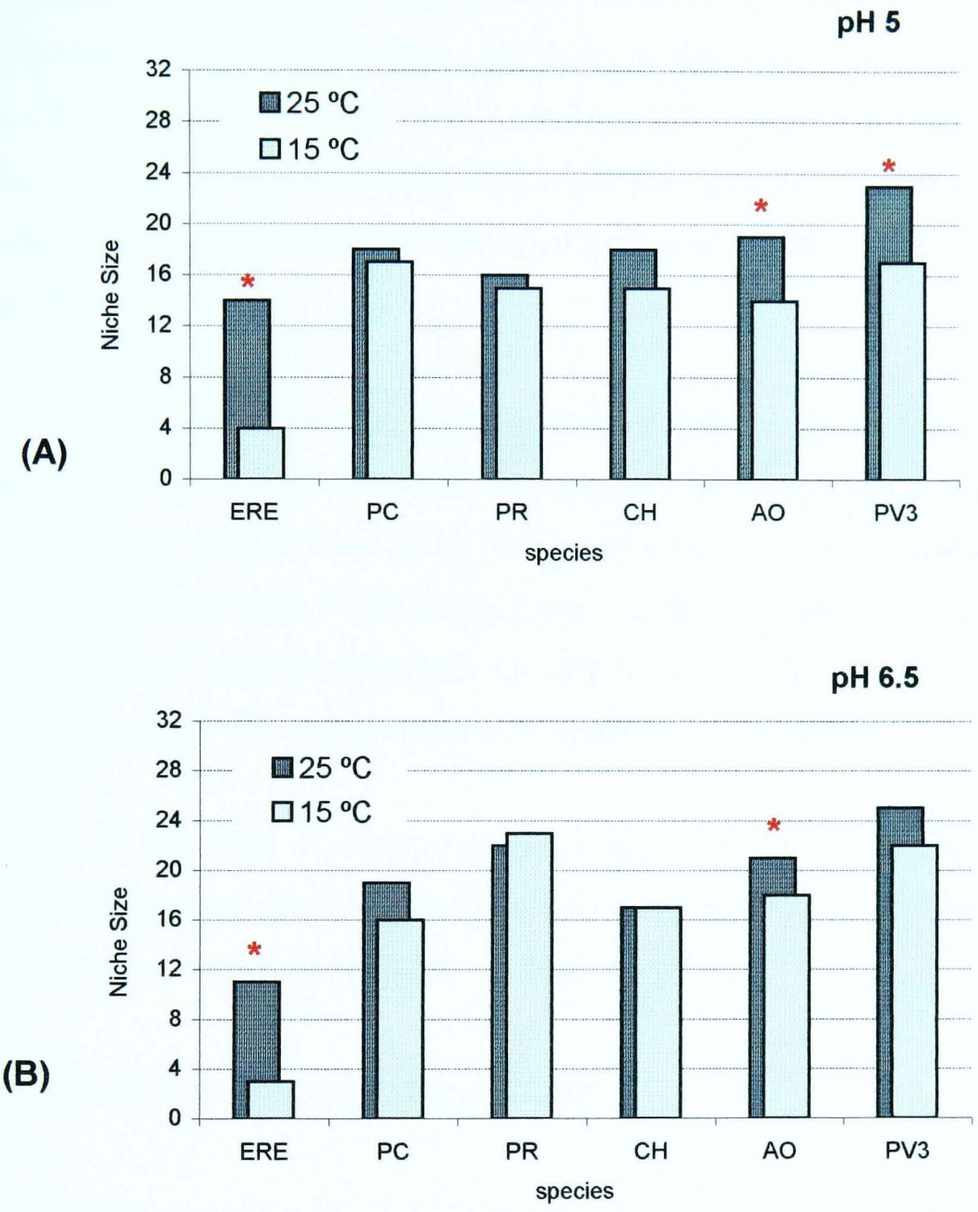


Figure 4.24 Effect of temperature on the niche size of *Eurotium repens* (ERE), *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR), *Cladosporium herbarum* (CH), *Aspergillus ochraceus* (AO) and strain PV3 of *Penicillium verrucosum* at 25°C, 0.97 a_w and (A) pH 5 and (B) pH 6.5. Asterisks show where the differences between temperatures are significant.

(ii) Effect of potassium sorbate

At pH 5, the addition of the organic acid increased the minimal a_w level for CS utilisation (see Table 4.10). In these conditions, no CS were used by any species at a_w values < 0.95, except *A.ochraceus* and *P.verrucosum* PV3 at 0.93 a_w and 25°C, although less than 3 CS were used. For all species, a general reduction of between 70-100% of the niche size occurred due to the addition of potassium sorbate at pH 5 and the higher a_w level, 0.97.

At pH 6.5, the minimal a_w was generally not affected by the addition of potassium sorbate. Moreover, at this pH level, the presence of preservative did not exert a significant effect ($p<0.05$) on niche sizes. However, for some species, especially *E.repens*, the total number of CS utilised was notably stimulated ($p< 0.05$). In Figure 4.25, the effect of potassium sorbate on niche sizes of *E.repens, A.ochraceus* and *P.verrucosum* strain PV3 is illustrated at different a_w and pH levels.

Table I-13 in Appendix I summarises the significance of a_w, pH, temperature and potassium sorbate and their two and three way interactions, on the number of total carbon sources utilised by the different species tested.

4.5.2 Effects on type of carbon source utilised

A much higher proportion of carbohydrates and amino acids than vitamins, over the total available in each group, was generally used. For instance, at the highest a_w level tested (0.97), all species used more than 11 of the possible 12 carbohydrates (>90%) while less than 2 of the 7 available vitamins (< 30%) regardless of pH and T, with or without potassium sorbate.

The proportion of amino acids used varied between species. For instance, at 0.97 a_w, less than 50% of amino acids (of the 13 available) were used by *E.repens*, *P.roquefortii* and *C.herbarum* while *A.ochraceus*, *P.corylophilum* and all strains of *P.verrucosum* used between 60 to 90%.

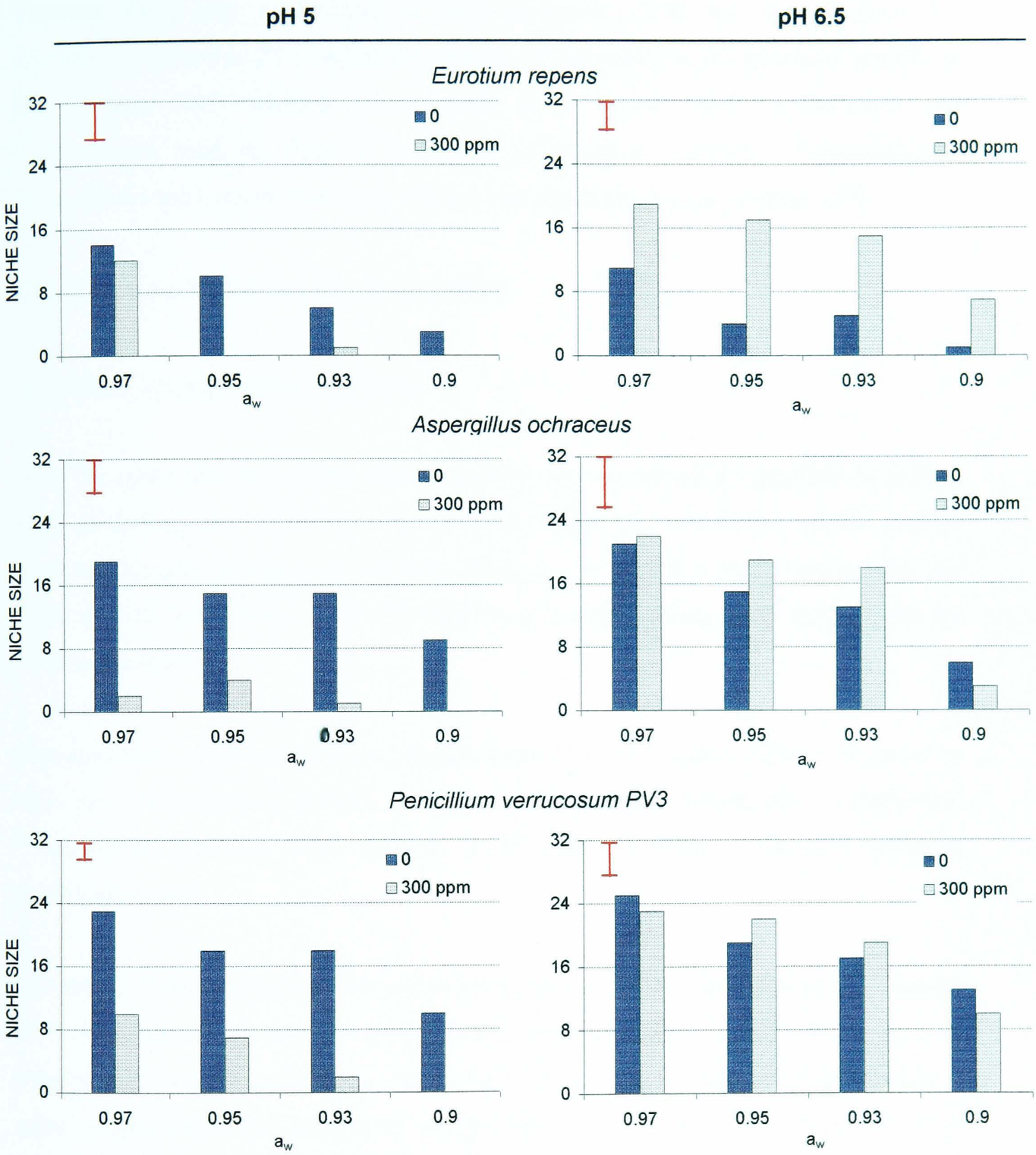


Figure 4.25 Effect of 300 ppm (w/w) of potassium sorbate on niche sizes of *E.repens*, *A.ochraceus* and *P.verrucosum* strain PV3 at different water activities (a_w) and pH levels. Bars indicate Least significant Differences (LSD) at $p < 0.05$.

Vitamins were only catabolised at high a_w levels (0.97 and 0.95) except for *P.verrucosum* (strains PV3 and M453) which, in the presence of potassium sorbate at pH 6.5 and 15°C, utilised 2-3 vitamins at a_w levels <0.95. *A.ochraceus* and *P.verrucosum* used the highest proportion of vitamins. *E.repens*, *P.corylophilum*, *P.roquefortii* and *C.herbarum*, only utilised some at the highest a_w studied, 0.97.

4.5.3 Effects on Niche Overlap Index (NOI)

(i) Effect of environmental conditions

From the niche size data, niche overlap indices were calculated as specified in section 3.8.3. At 0.97 a_w, pH 5 and 25°C, *P.verrucosum*, strains PV3 and M453, was the species most nutritionally competitive, showing $NOI_{spp/PV3}$ values >0.9 when paired with any other species (i.e. >90% of the CS used by a certain species were also utilised by *P.verrucosum* PV3).

Comparatively, values of $NOI_{PV3/spp}$ ranged from 0.57 (57% shared CS) for *E.repens* to 0.87 for *P.verrucosum* M450. At these environmental conditions, *C.herbarum*, *P.roquefortii*, *E.repens*, *P.verrucosum M450* and *A.ochraceus* occupied different nutritional niches.

Interactions between species changed as the a_w was reduced. The nutritional advantage of *P.verrucosum* (Pv) over the other species was generally increased as the a_w was lowered, with increasing $NOI_{spp/Pv.}$ and decreasing $NOI_{Pv/spp}$ values. Moreover, at lower water availabilities the nutritional competition of *E.repens* and *C.herbarum* was reduced. In fact, while at 0.97 a_w they occupied separate niches from all species except *P.verrucosum* PV3, at a_w <0.95 all species occupied the same niche with $NOI_{CH/spp}$ and $NOI_{ERE/spp}$ values of 1. This effect was more marked at 15°C. Table 4.11 depicts the NOI between *P.verrucosum* PV3 when paired with the other test species at 15°C at the different a_w and pH levels tested.

Table 4.11 Niche Overlap Index for different species paired with *Penicillium verrucosum* strain PV3 at 15°C and different a_w and pH levels.

15°C - pH 5

a_w	0.97		0.95		0.93		0.90	
P.verrucosum PV3	NS_{PV3} 17		NS_{PV3} 17		NS_{PV3} 14		NS_{PV3} 11	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	4	0.12/0.5	2	0.12/1	2	0.14/1	0	0/0
P.corylophilum	17	0.94/0.94	14	0.76/0.93	11	0.79/1	6	0.55/1
C.herbarum	15	0.71/0.8	12	0.71/1	0	0/0	0	0/0
P.roquefortii	15	0.76/0.87	8	0.47/1	0	0/0	0	0/0
A.ochraceus	14	0.71/0.86	6	0.35/1	10	0.71/1	1	0.09/1
P.verrucosum M450	18	0.94/0.89	16	0.88/0.94	17	0.93/0.76	13	0.91/0.77
P.verrucosum M453	20	1/0.85	16	0.94/1	16	1/0.88	15	1/0.73

15°C - pH 6.5

a_w	0.97		0.95		0.93		0.90	
P.verrucosum PV3	NS_{PV3} 22		NS_{PV3} 16		NS_{PV3} 14		NS_{PV3} 9	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	3	0.14/1	1	0.06/1	2	0.14/1	0	0/0
P.corylophilum	16	0.68/0.8	4	0.25/1	4	0.21/0.75	0	0/0
C.herbarum	17	0.64/0.82	11	0.63/0.91	0	0/0	0	0/0
P.roquefortii	23	0.91/0.87	3	0.19/1	1	0.07/1	0	0/0
A.ochraceus	18	0.77/0.94	5	0.31/1	6	0.36/0.83	0	0/0
P.verrucosum M450	23	1/0.96	17	1/0.94	13	0.79/0.85	4	0.33/0.75
P.verrucosum M453	21	0.91/0.72	18	0.94/0.83	13	0.71/0.77	13	0.89/0.62

NS: niche size
NOI_{PV3} x 100: % of CS used by *P.verrucosum* PV3 that were also used by the competitive species
NOI_{spp} x 100: % of CS used by the competitive species that were also used by *P.verrucosum* PV3

At pH 6.5 similar trends to those shown at the lower pH were observed for *P.verrucosum* strains. On the other hand and contrary to that recorded at pH 5, *E.repens* occupied separate niches as the a_w was reduced below 0.95 although its niche size was only 1 CS.

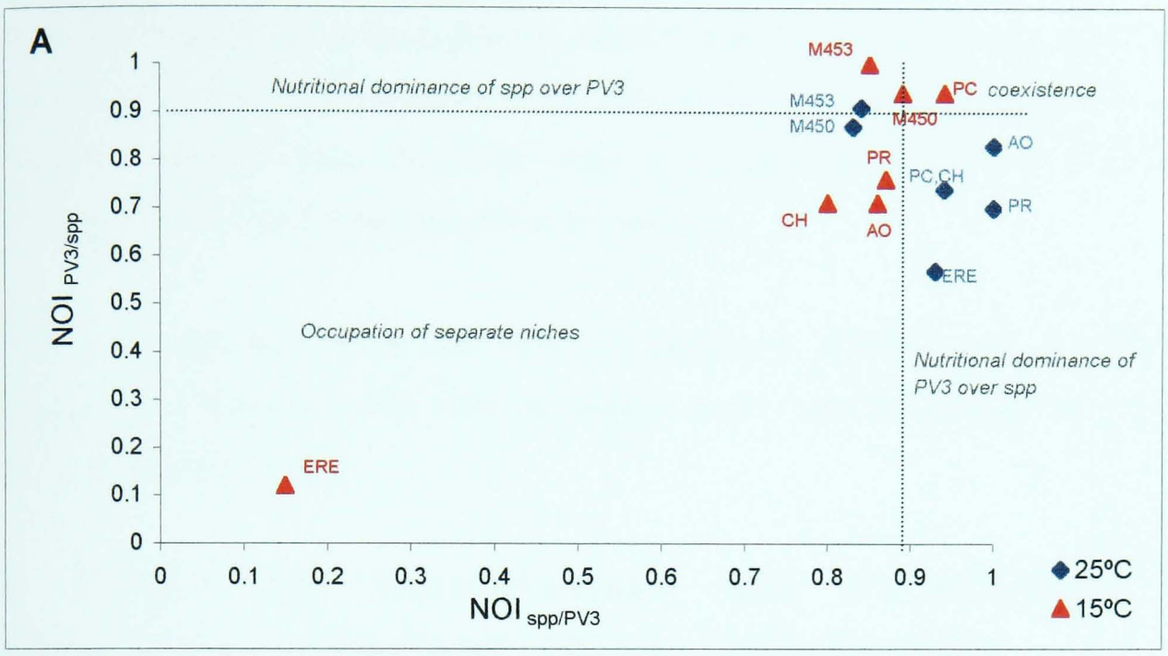
At 15°C, interactions between certain species were markedly different. At 25°C and pH 5, for instance, *A.ochraceus* was nutritionally favoured over *P.corylophilum* with $NOI_{AO/PC}$ / $NOI_{PC/AO}$ values of 0.84/0.94. However, when the temperature was reduced to 15°C, values of 0.93/0.76 were recorded. This indicated that, while still in the same nutritional niche, a change in the favoured species occurred. Furthermore, at 0.97 a_w, the temperature reduction resulted in *P.verrucosum* occupying separate niches from the rest of species and to coexist with *P.corylophilum.* In Figure 4.26, the effect of temperature on niche interactions between *P.verrucosum* PV3 and *A.ochraceus* and the other test species is illustrated at 0.97a_w and pH 5.

(ii) Effect of potassium sorbate

The effect of sub-optimal concentrations of potassium sorbate on fungal interactions was again dependent upon the pH of the media. At pH 5, the addition of 300 ppm (w/v) of potassium sorbate resulted in *P.verrucosum* being more nutritionally favoured over all species studied (with the exception of *E.repens* at 0.97 a_w) with $NOI_{PV3/spp}$=0.2-0.7 and $NOI_{spp/PV3}$ =1 regardless of temperature and a_w levels. At 0.97 a_w, pH 5 and 25°C, the addition of 300ppm potassium sorbate separated *E.repens* and *P.verrucosum* nutritional niches with less than 60% relative niche overlap.

At pH 6.5 and 25°C, potassium sorbate increased the nutritional competition and coexistence. *A.ochraceus* changed from occupying separate niches from most of the species with 72-89% niche overlap when no preservative was added, to coexist with *P.verrucosum* PV3 and *P.roquefortii* and to nutritionally dominate over *P.corylophilum* and *C.herbarum* in the presence of potassium sorbate.

Target pathogen: *Penicillium verrucosum* PV3
0.97a_w and pH 5



Target pathogen: *Aspergillus ochraceus*
0.97a_w and pH 5

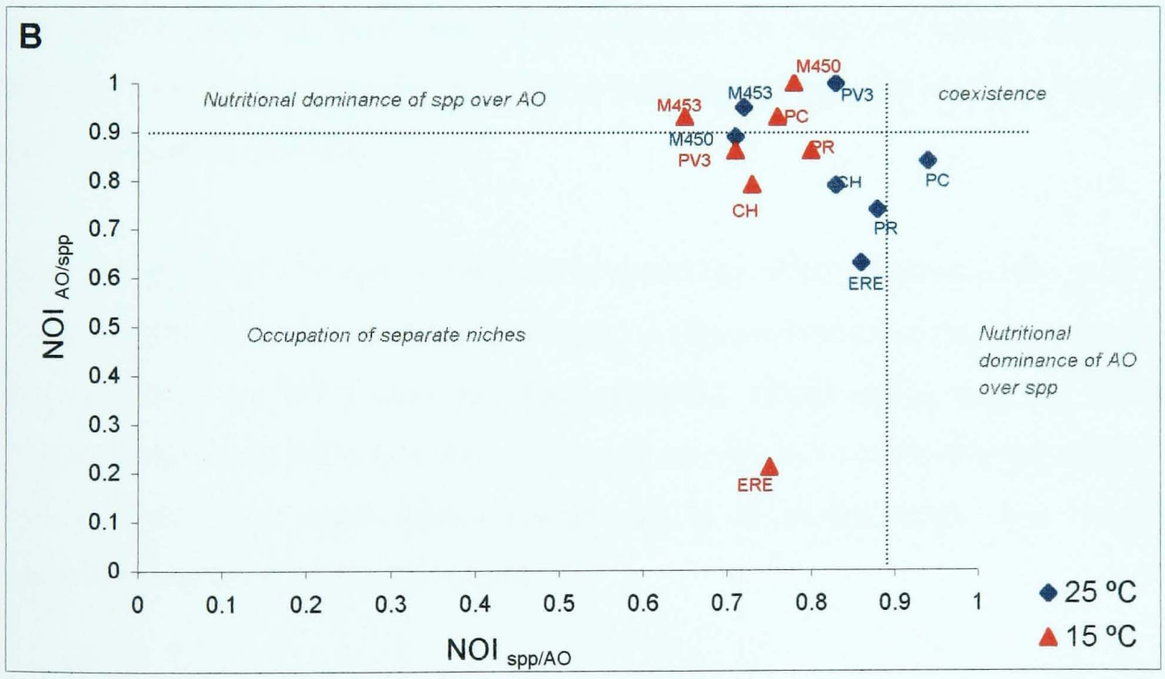


Figure 4.26 Effect of temperature on species interaction between (A) *Penicillium verrucosum* strain PV3 (PV3) and (B) *Aspergillus ochraceus* (AO) paired with *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR), *Penicillium verrucosum* (strains M450 and M453), *Eurotium repens* (ERE) and *Cladosporium* at 0.97 a_w and pH 5.

The increase in competition for nutrients was particularly noticeable at 15°C where *C.herbarum* was able to coexist with *A.ochraceus*, *P.roquefortii* and *P.verrucosum* (strains M453 and PV3) at the highest a_w studied, 0.97. Figure 4.27 compares the niche interactions between *A.ochraceus* and other test species in media containing 300 ppm of potassium sorbate to those occurring when no preservative is added. The effect is illustrated at $0.95a_w$ and at the two pH levels studied.

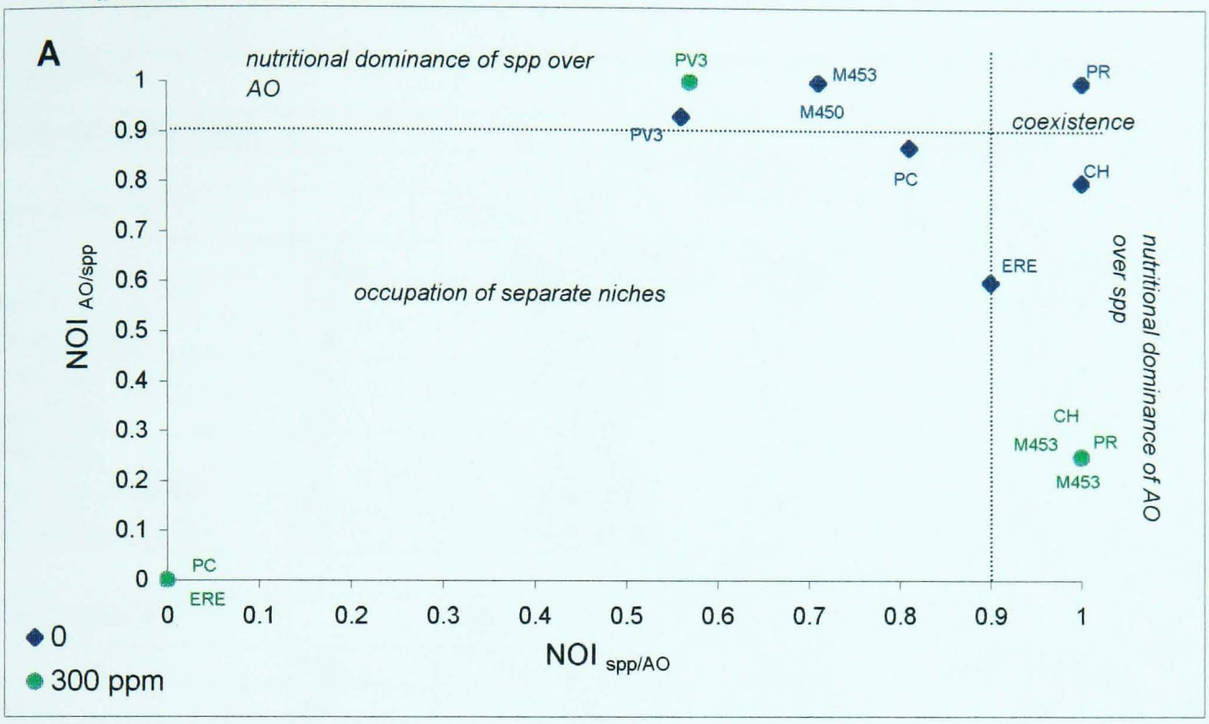
Tables 4.12 and 4.13 show the effect of potassium sorbate on NOI values of *P.verrucosum* PV3 paired with other test species at pH 5 and 6.5 respectively, at 25°c and the different a_w levels.

4.6 IMPACT OF PRESERVATIVES AND ENVIRONMENT ON HYDROLYTIC ENZYME PRODUCTION BY SPOILAGE FUNGI

The final set of experiments of this project was carried out on the effect of existing and novel preservatives and environmental factors on the temporal hydrolytic enzyme production on both 2% WFA and bread analogues in order to identify whether the efficacy of a certain preservative was due to inhibition of specific enzymes involved in fungal colonisation of bread.

Preservative doses of 150 ppm were used to assess the effect of preservative type on *in vitro* mould enzyme production profiles since, at this concentration significant levels of growth inhibition on WFA were generally recorded. Additionally, doses of 300 ppm were also analysed on WFA at 0.97 a_w and pH 6 to establish a point of comparison with *in situ* studies where much higher concentrations of preservatives were needed to achieve the same level of growth control.

Target pathogen: *Aspergillus ochraceus*
0.95 a_w and pH 5



Target pathogen: *Aspergillus ochraceus*
0.95 a_w and pH 6.5

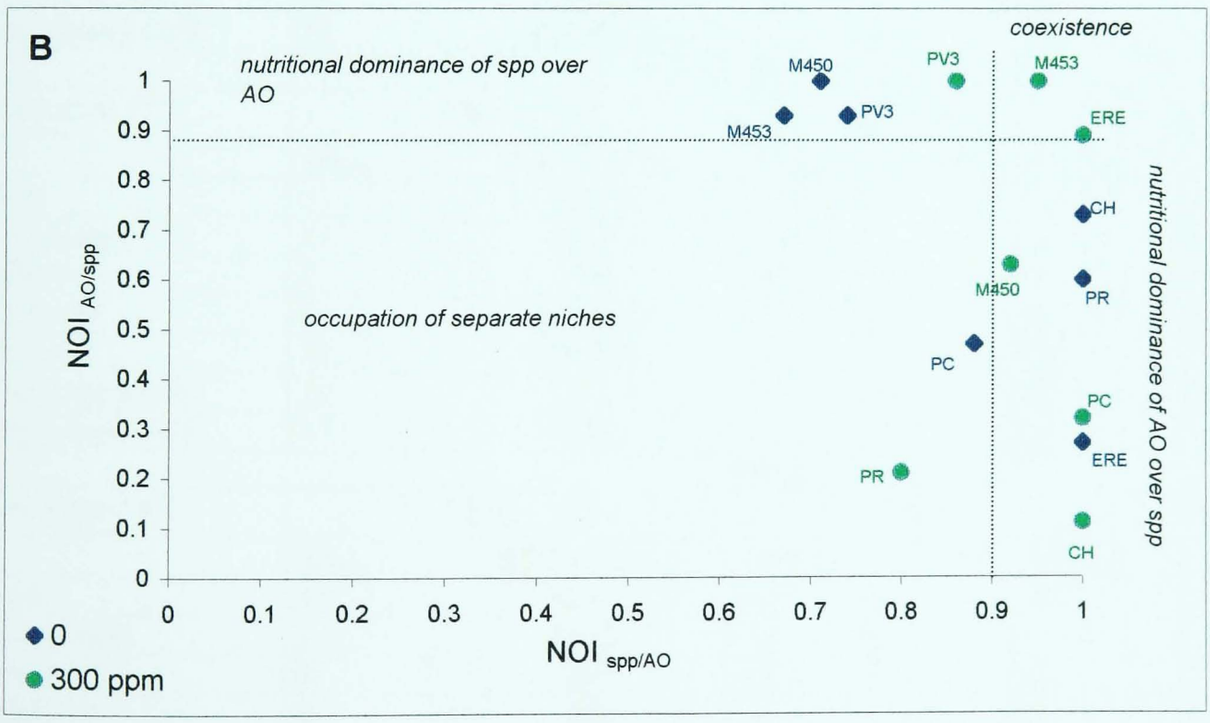


Figure 4.27 Effect of 300 ppm (w/w) of potassium sorbate on species interaction between *Aspergillus ochraceus* (AO) and *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR), *Penicillium verrucosum* (strains PV3, M450 and M453), *Eurotium repens* (ERE) and *Cladosporium* at 0.95 a_w and (A) pH 5 and (B) pH 6.5.

Table 4.12 Comparison of NOI values of different species paired with *P. verrucosum* PV3 in the presence or absence of potassium sorbate (PS) at 25°C, pH 5 and different a_w levels.

pH 5 and 25°C

Concentration PS (w/v)	**0**		**300 ppm**	
		0.97 a_w		
P.verrucosum PV3	NS_{PV3} 23		NS_{PV3} 10	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*14*	0.57/0.93	*12*	0.6/0.5
P.corylophilum	*18*	0.74/0.94	*3*	0.3/1
C.herbarum	*18*	0.74/0.94	*1*	0.1/1
P.roquefortii	*16*	0.7/1	*8*	0.7/1
A.ochraceus	*19*	0.83/1	*2*	0.2/1
P.verrucosum M450	*24*	0.87/0.83	*4*	0.4/1
P.verrucosum M453	*25*	0.91/0.84	*4*	0.4/1
		0.95 a_w		
P.verrucosum PV3	NS_{PV3} 18		NS_{PV3} 7	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*10*	0.56/1	*0*	0/0
P.corylophilum	*16*	0.78/0.88	*0*	0/0
C.herbarum	*12*	0.67/1	*1*	0.14/1
P.roquefortii	*8*	0.44/1	*1*	0.14/1
A.ochraceus	*15*	0.78/0.93	*4*	0.57/1
P.verrucosum M450	*21*	0.94/0.81	*1*	0.14/1
P.verrucosum M453	*21*	0.89/0.76	*1*	0.14/1
		0.93 a_w		
P.verrucosum PV3	NS_{PV3} 18		NS_{PV3} 2	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*6*	0.33/1	*1*	0/0
P.corylophilum	*13*	0.72/1	*0*	0/0
C.herbarum	*0*	0/0	*0*	0/0
P.roquefortii	*9*	0.44/0.89	*0*	0/0
A.ochraceus	*15*	0.83/1	*1*	0.5/1
P.verrucosum M450	*18*	1/1	*0*	0/0
P.verrucosum M453	*17*	0.94/1	*0*	0/0
		0.90 a_w		
P.verrucosum PV3	NS_{PV3} 10		NS_{PV3} 0	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*3*	0.3/1	*0*	0/0
P.corylophilum	*5*	0.5/1	*0*	0/0
C.herbarum	*0*	0/0	*0*	0/0
P.roquefortii	*0*	0/0	*0*	0/0
A.ochraceus	*9*	0.9/1	*0*	0/0
P.verrucosum M450	*10*	1/1	*0*	0/0
P.verrucosum M453	*12*	1/0.83	*0*	0/0

NS: niche size

NOI_{PV3} x 100: % of CS used by *P.verrucosum* PV3 that were also used by the paired species

NOI_{spp} x 100: % of CS used by the paired species that were also used by *P.verrucosum* PV3

Table 4.13 Comparison of NOI values of different species paired with *P.verrucosum* PV3 in the presence or absence of potassium sorbate (PS) at 25°C, pH 6.5 and different a_w levels.

25°C –pH 6.5

Concentration PS (w/v)	**0**		**300 ppm**	
		0.97 a_w		
P.verrucosum PV3	NS_{PV3} 25		NS_{PV3} 23	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*11*	0.4/0.91	*19*	0.78/0.95
P.corylophilum	*19*	0.76/1	*21*	0.87/0.95
C.herbarum	*17*	0.68/1	*14*	0.61/1
P.roquefortii	*22*	0.8/0.91	*20*	0.87/1
A.ochraceus	*21*	0.72/0.86	*22*	0.91/0.95
P.verrucosum M450	*21*	0.84/1	*19*	0.83/1
P.verrucosum M453	*22*	0.88/1	*24*	1/0.96
		0.95 a_w		
P.verrucosum PV3	NS_{PV3} 19		NS_{PV3} 22	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*4*	0.21/1	*17*	0.77/1
P.corylophilum	*8*	0.37/0.88	*6*	0.27/1
C.herbarum	*11*	0.58/1	*2*	005/1
P.roquefortii	*8*	0.42/1	*5*	0.18/0.8
A.ochraceus	*15*	0.74/0.93	*16*	0.86/1
P.verrucosum M450	*21*	1/0.9	*13*	0.55/0.92
P.verrucosum M453	*21*	1/0.9	*20*	0.91/1
		0.93 a_w		
P.verrucosum PV3	NS_{PV3} 17		NS_{PV3} 19	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*5*	0.18/0.6	*15*	0.74/0.93
P.corylophilum	*3*	0.12/0.67	*1*	0.05/1
C.herbarum	*0*	0/0	*0*	0/0
P.roquefortii	*2*	0.12/1	*0*	0/0
A.ochraceus	*13*	0.71/0.92	*18*	0.95/1
P.verrucosum M450	*16*	0.76/081	*12*	0.63/1
P.verrucosum M453	*13*	0.76/1	*14*	0.74/1
		0.90 a_w		
P.verrucosum PV3	NS_{PV3} 13		NS_{PV3} 10	
	NS_{spp}	NOI_{PV3}/NOI_{spp}	NS_{spp}	NOI_{PV3}/NOI_{spp}
E.repens	*1*	0/0	*7*	0.3/0.43
P.corylophilum	*2*	0.08/0.5	*0*	0/0
C.herbarum	*0*	0/0	*0*	0/0
P.roquefortii	*0*	0/0	*1*	0/0
A.ochraceus	*6*	0.46/1	*3*	0.2/0.67
P.verrucosum M450	*2*	0.15/1	*2*	0.2/1
P.verrucosum M453	*5*	0.31/0.8	*4*	0.4/1

NS: niche size

NOI_{PV3} x 100: % of CS used by *P.verrucosum* PV3 that were also used by the paired species

NOI_{spp} x 100: % of CS used by the paired species that were also used by *P.verrucosum* PV3

4.6.1 Enzyme activity on WFA in the absence of preservatives

Initially, 7 different hydrolytic enzymes involved in basic metabolic pathways were considered for the study (see Table 3.9, page 72). An initial screen of the level of activity of all enzymes was conducted at 0.97 a_w. Of the seven enzymes, only α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase were produced at detectable levels. Subsequent experimentation was focused on the effect of preservation factors on total and specific activity of these three enzymes. Enzymatic activity was determined during the first 12 days of mould growth (see section 3.9.1)

Figure 4.28 illustrates the temporal changes in total and specific activity for the three enzymes in growing cultures of *P.roquefortii, E.repens* and *P.verrucosum* (strain M453) at 0.97 a_w, pH 6 with no added preservative.

N-acetyl-β-D-glucosaminidase was the enzyme that showed the highest levels of activity over all treatments and for all species except for *P.roquefortii.* In contrast, α-D-galactosidase was produced in the lowest amounts. The production of β-D-glucosidase appeared to be species dependent. In *Aspergillus* and *Eurotium* isolates for instance, no significant differences between activity levels of β-D-glucosidase and α-D-galactosidase were observed ($p>0.05$) while in *Penicillium* isolates (except for *P.roquefortii*) the same total and specific activity profiles for N-acetyl-β-D-glucosaminidase were obtained ($p>0.05$).

In cultures of *P.roquefortii* significant differences were observed between all enzymes with β-D-glucosidase showing the highest activities. Detectable levels of activity were found from the 3-6 day incubation achieving maximum levels after 9-12 days. Overall, total and specific activities followed similar patterns. Total protein content significantly increase with time ($p<0.001$) with maximum levels of 5μg for *Penicillium* and *Eurotium* isolates and up to 15μg in cultures of *Aspergillus ochraceus* (Figure 4.29).

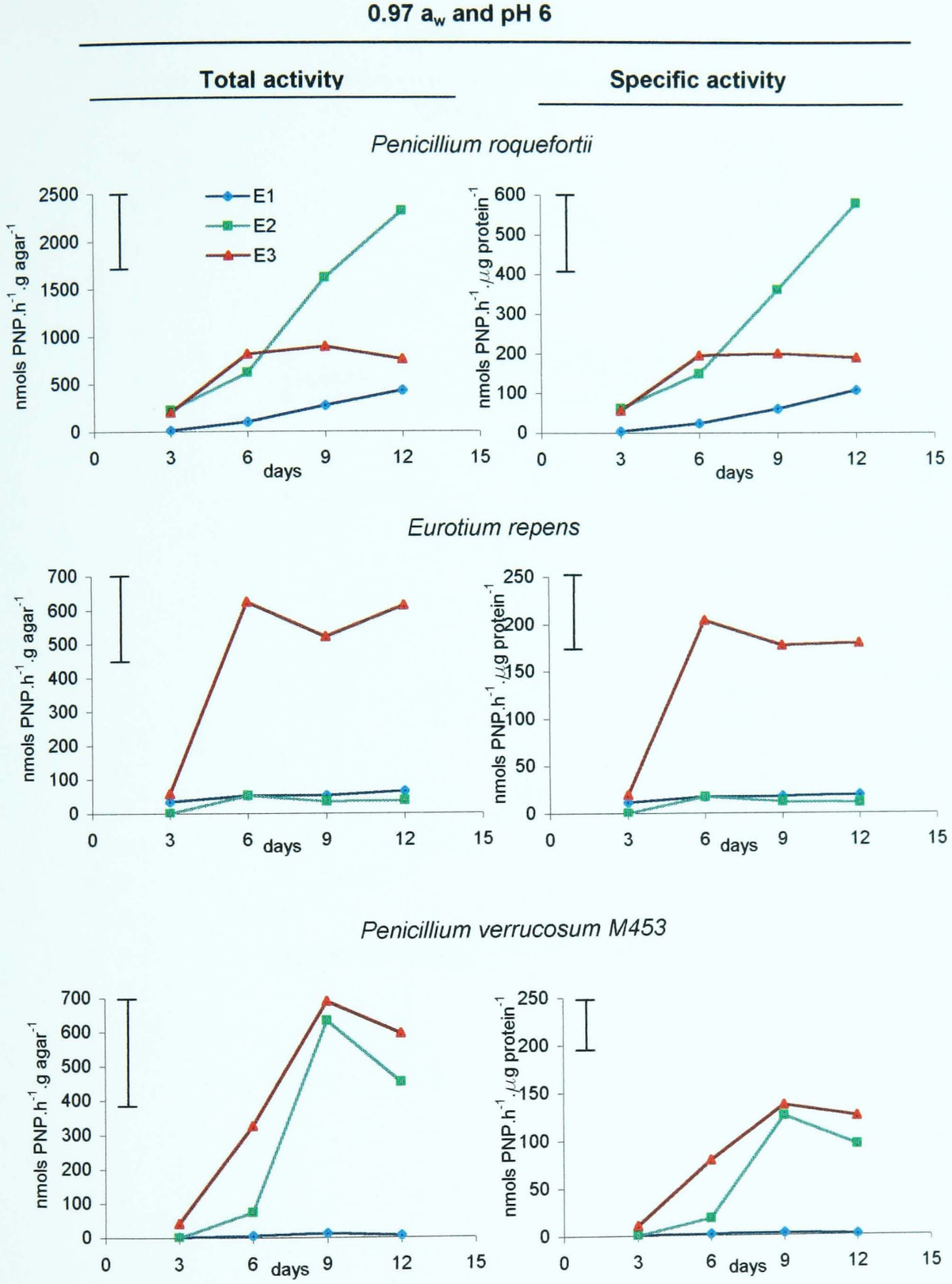


Figure 4.28 Total and specific activity profiles of α-D-Galactosidase (E1), β-D-glucosidase (E2) and N-acetyl-β-D-glucosaminidase (E3) in cultures of *Penicillium roquefortii*, *Eurotium repens* and *Penicillium verrucosum* (strain M453) on 2% wheat flour agar, at pH 6 and 0.97a_w. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

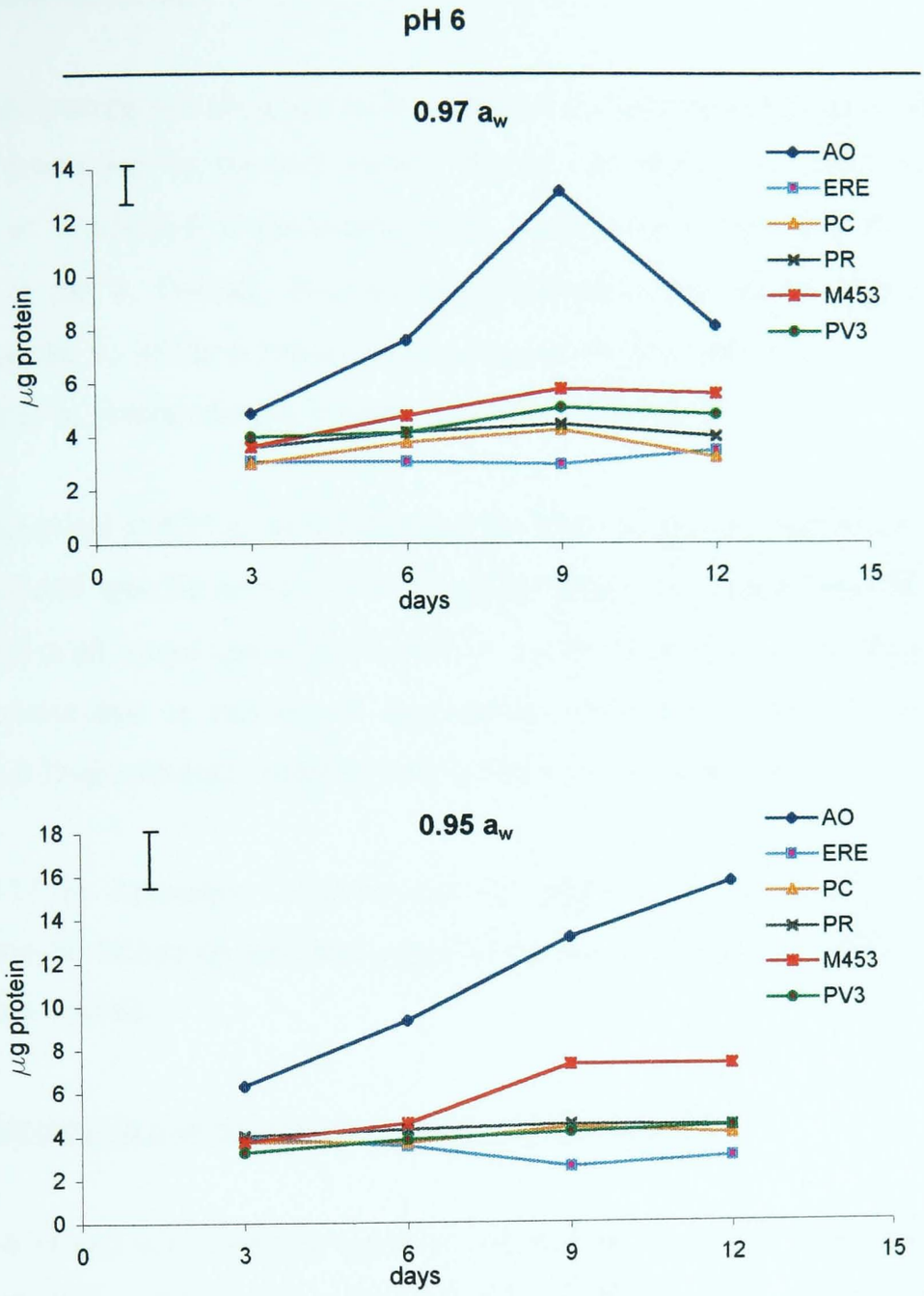


Figure 4. 29 Total protein contents (μg) profiles in cultures of *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR) and *Penicillium verrucosum* strains M453 and PV3 growing on 2% wheat flour agar at pH 6 and 0.97-0.95 a_w. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

(i) Effect of a_w and pH

No general pattern was observed on the effect of a_w on temporal enzyme production for enzyme production by the test species. Figure 4.30 shows the effect of a_w on total activity of N-acetyl-β-D-glucosaminidase produced by *Eurotium* and *Penicillium* isolates at pH 6. Overall, N-acetyl-β-D-glucosaminidase showed higher levels of activity as the a_w of the substrate was increased. On the other hand, β-D-glucosidase appeared to be produced more at lower a_w levels of 0.93-0.95.

The effect of pH at 0.95 a_w also influenced the type of enzyme produced by the species. Both total and specific activity of α-D-galactosidase were significantly higher at pH 6 than at 4.5 in all fungal species ($p<0.001$). A similar effect was observed for N-acetyl-β-D-glucosaminidase in cultures of *A.ochraceus, P.roquefortii* and *P.verrucosum.* In contrast, β-D-glucosidase appeared to be more active at a lower pH.

Table I-14 in Appendix I summarises the analysis of variance on the effect of environmental factors on total and specific enzyme activity and on protein contents for the species studied.

(ii) Differences in enzyme activity between species on WFA

Figures 4.31 and 4.32 compare the levels of total activity of all three enzymes and all species studied at $0.95a_w$ and pH 6 and 4.5 respectively. The differences in enzyme production between species varied with the type of enzyme. Overall, *A.ochraceus* and *P.roquefortii* were the species with higher levels of enzyme detected. Both species produced significantly higher levels ($p<0.001$) of total and specific activity of α-D-galactosidase compared to other tested species. Production of α-D-galactosidase by *P.roquefortii* was stimulated at high a_w (0.97), low pH (4.5) and long incubation time (9-12 days). On the other hand, *A.ochraceus* showed the highest activity of N-acetyl-β-D-glucosaminidase. Similar and greater extracellular enzyme was produced in cultures of *P.corylophilum* at pH 4.5 after 9-12 days incubation.

Total activity of N-acetyl-β-D-glucosaminidase at pH 6

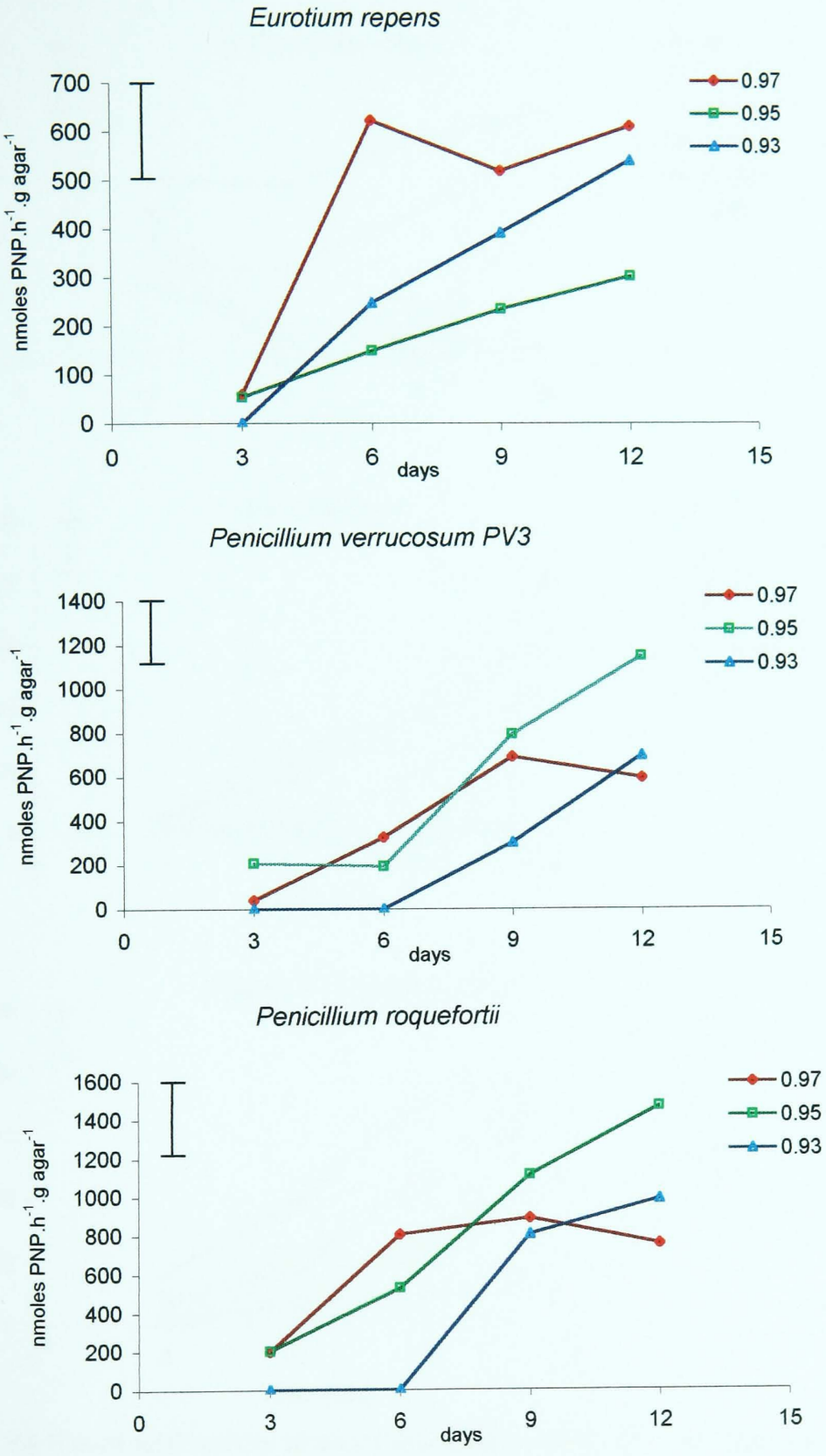


Figure 4.30 Effect of water activity (a_w) on total activity of N-acetyl-β-D-glucosaminidase in cultures of *Eurotium repens*, *Penicillium verrucosum* (strain PV3) and *Penicillium roquefortii* on 2% wheat flour agar at pH 6. Bars represent Least Significant Differences (LSD) for $p < 0.05$.

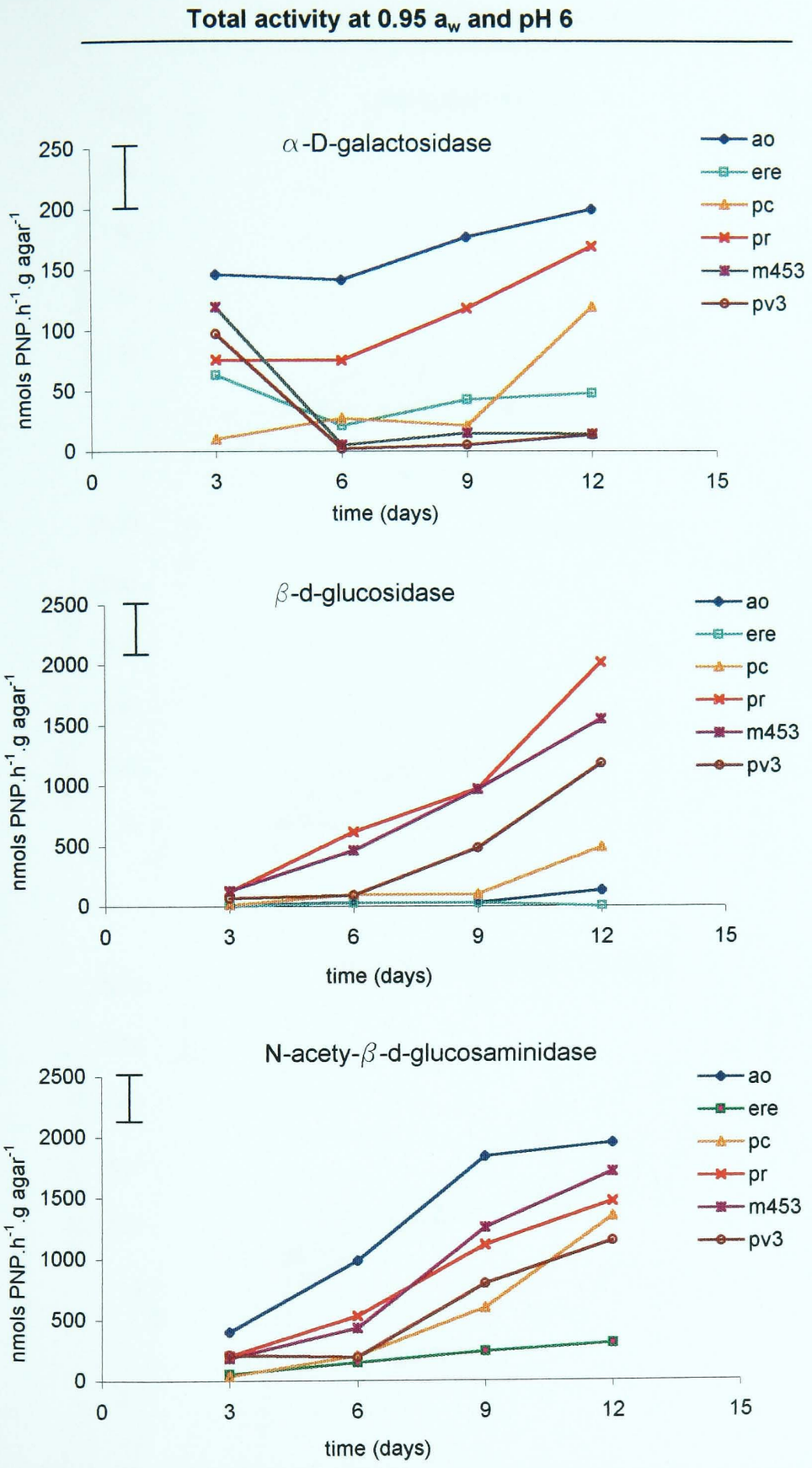


Figure 4.31 Profiles of total activity levels of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase on cultures of *Aspergillus ochraceus* (ao), *Eurotium repens* (ere), *Penicillium corylophilum* (pc), *Penicillium roquefortii* (pr) *and Penicillium verrucosum* strains PV3 (pv3) and M453 (m453) growing on 2% wheat flour agar, 0.95a_w and pH 6. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

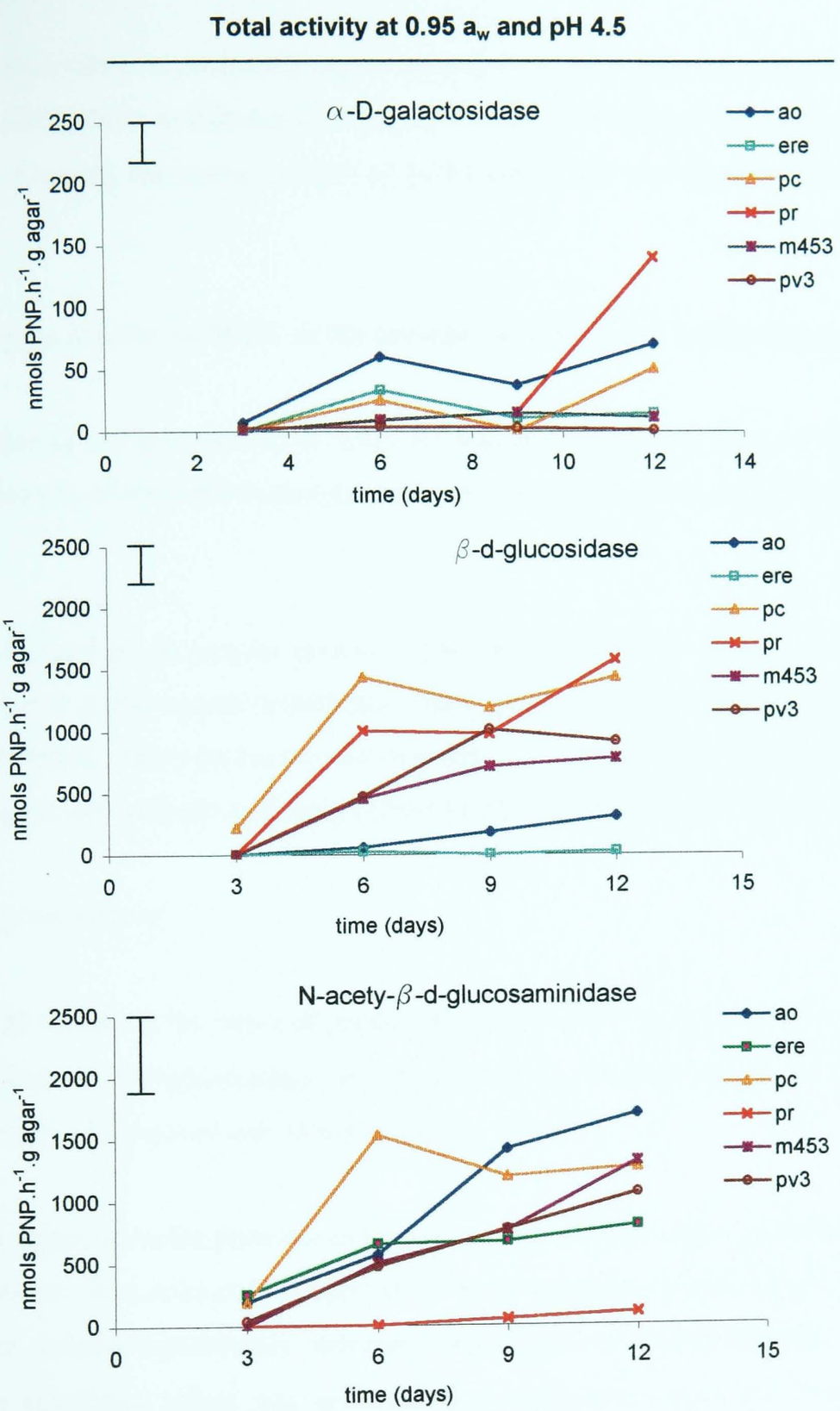


Figure 4.32 Profiles of total activity levels of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase on cultures of *Aspergillus ochraceus* (ao), *Eurotium repens* (ere), *Penicillium corylophilum* (pc), *Penicillium roquefortii* (pr) *and Penicillium verrucosum* strains PV3 (pv3) and M453 (m453) growing on 2% WFA, 0.95a_w and pH 4.5. Bars indicate Least Significant Differences (LSD) at $p< 0.05$.

For β-D-glucosidase, significantly higher activity by *Penicillium* isolates ($p<0.001$) was obtained, particularly in cultures of *P.roquefortii* for all treatments and *P.corylophilum* at pH 4.5. Overall, the lowest activity of β-D-Glucosidase was observed in cultures of *E.repens*.

4.6.2 Enzyme activity on WFA in the presence of 150ppm of different preservatives

The addition of any preservative at doses of 150ppm significantly affected the total and specific activity of the three hydrolytic enzymes ($p<0.001$) when compared with control plates.

Effects of a_w and pH on enzyme activity in the presence of preservatives were similar to those reported in the control treatments. Interestingly, different types of preservatives exerted different effects on enzyme activity dependent upon the species and treatment. Following are outlined some of these effects for each group of preservatives studied.

(i) Potassium sorbate

Figure 4.33 illustrates the effect of potassium sorbate on the levels of total activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase in cultures of *A.ochraceus* and *P.verrucosum* M453 at 0.97 a_w and pH 6.

Different responses to the presence of potassium sorbate were observed between species and treatments. In *A.ochraceus* isolates at pH 6, for example, the addition of 150 ppm of potassium sorbate significantly increased production of β-D-glucosidase ($p<0.001$) while no significant effect was observed for α-D-galactosidase and N-actetyl-β-D-glucosaminidase. In contrast, other fungal isolates such as *E.repens* or *P.verrucosum* showed greater levels of α-D-galactosidase in the presence of preservative than the controls.

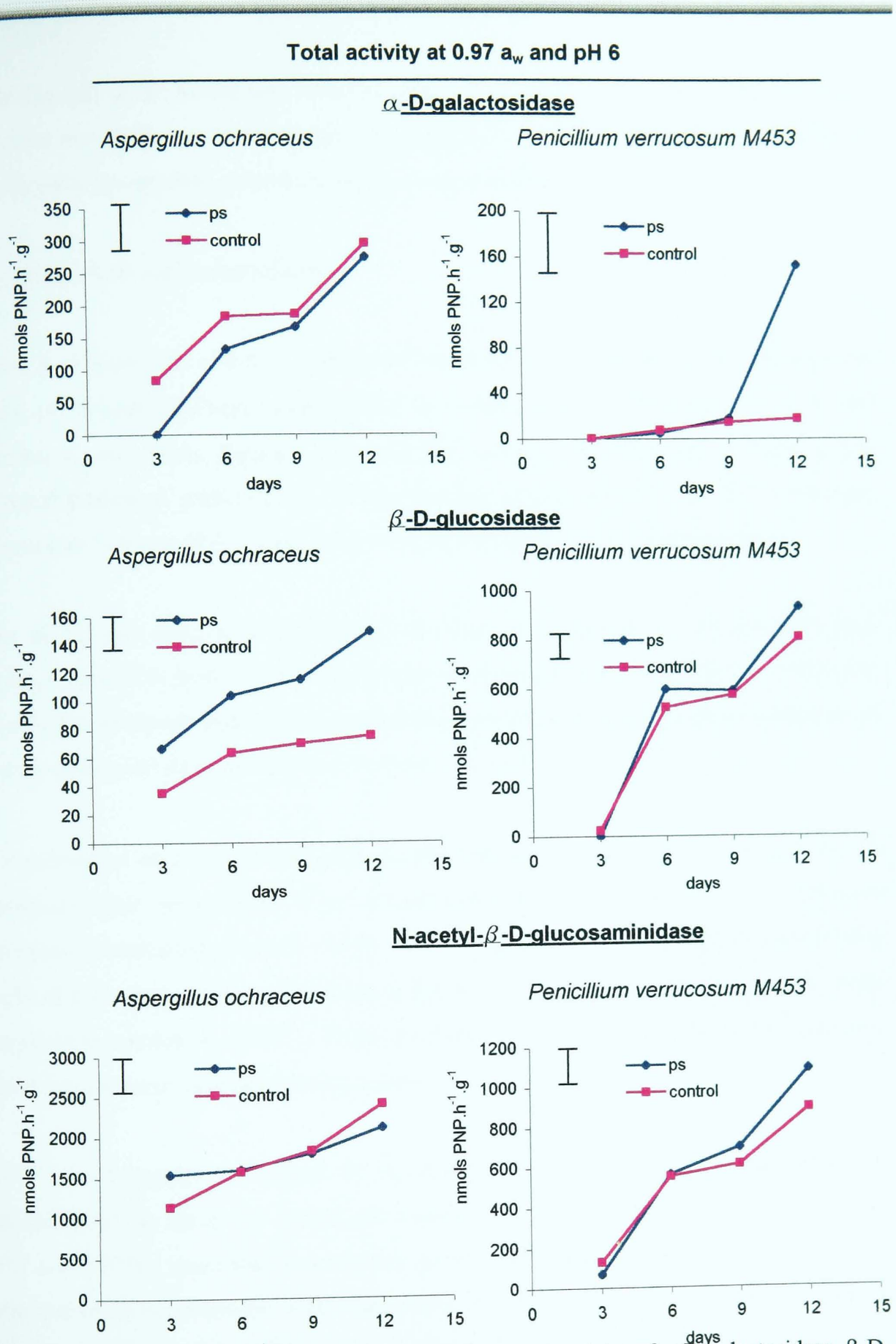


Figure 4.33 Effect of 150 ppm of potassium sorbate on total activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase on cultures of *Aspergillus ochraceus* and *Penicillium verrucosum* (strain M453) on 2 % wheat flour agar, at 0.97a_w and pH 6. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

When the pH of the media was lowered to pH 4.5 a significant reduction of all enzyme activities was observed. Interestingly, in all cases, total protein contents were similar or significantly lower when potassium sorbate was added to the substrate.

(ii) Antioxidants and essential oils

Figure 3.34 shows the effect of 150ppm of antioxidants and essential oils on production levels of N-acetyl-β-D-glucosaminidase by cultures of *A.ochraceus* at pH 6 and different a_w treatments. Figures 4.35 and 4.36 compare the effect of 150 ppm of the different antifungal preservatives on the specific activity of the three key hydrolytic enzymes on cultures of *P.roquefortii* at 0.95 a_w and pH 6 and 4.5 respectively

Over all a_w and species, a significant reduction of production of all three enzymes occurred when 150 ppm of BHA or propyl paraben were added to the media. Similarly, the addition of thyme and cinnamon essential oils resulted in a significant reduction of all enzymatic activity in cultures of *E.repens. P.corylophilum* and *P.verrucosum.*

In *A.ochraceus* at pH 6, stimulation of total and specific activities of N-acetyl-β-D-glucosaminidase were found when thyme essential oil was added to the substrate compared to untreated controls. At pH 4.5, *P.roquefortii* produced significantly higher levels of β-D-glucosidase and α-D-glucosidase in the presence of cinnamon oil when compared to controls ($p<0.001$). These increases in activity were generally related to a significant increase in total protein content.

Table 4.14 compiles the effect of all preservatives (existing and alternative) on production of the three key hydrolytic enzymes by all species on wheat flour agar at 25°C and 0.95 a_w compared to controls. Analysis of variance on the effect of type of preservative in combination with environmental factors on total and specific enzyme activity is summarised in Tables I-15a, I-15b and I-15c (Appendix I)

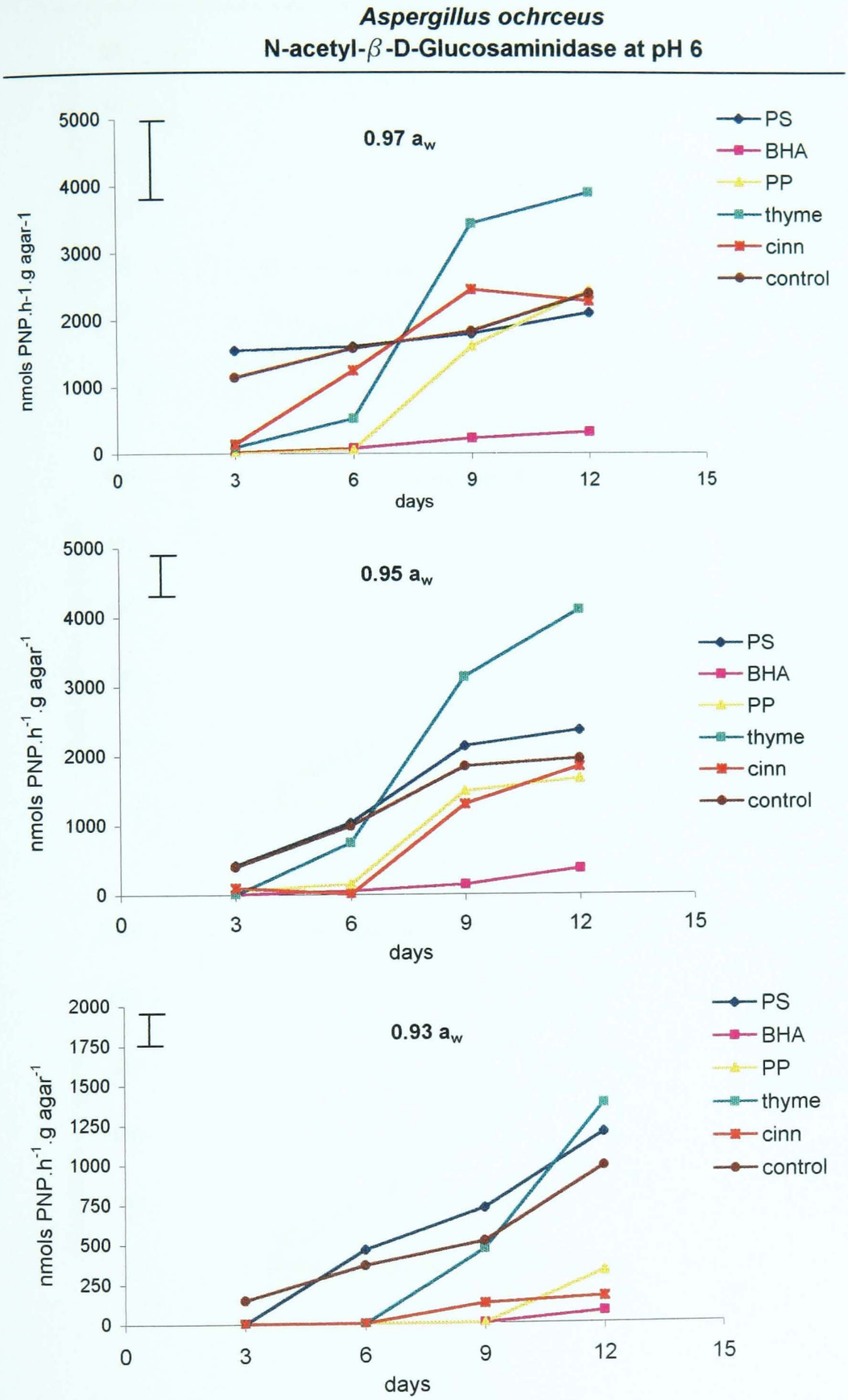


Figure 4.34 Effect of 150 ppm of potassium sorbate (PS), BHA, propyl paraben (PP), thyme and cinnamon (cinn) essential oils on total activity profiles of N-acetyl-β-D-glucosaminidase in cultures of *Aspergillus ochraceus* growing on 2% wheat flour agar at pH 6 and different water activity (a_w) levels. Bars indicate Least Significant Differences at $p < 0.05$.

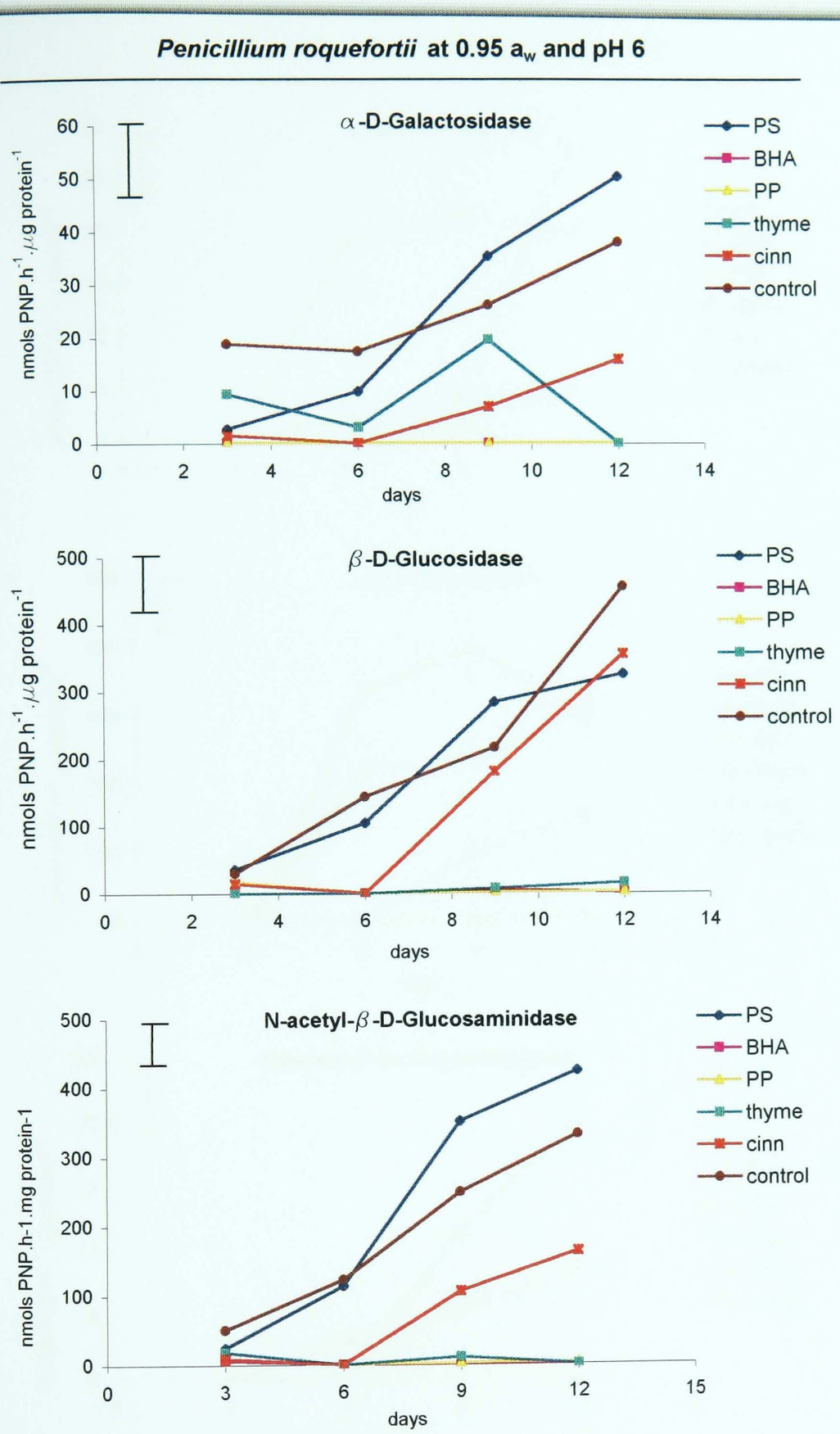


Figure 4.35 Effect of 150 ppm of potassium sorbate (PS), BHA, propyl paraben (PP), thyme and cinnamon (cinn) essential oils on specific activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase from cultures of *Penicillium roquefortii* on 2% wheat flour agar, at 0.95 a_w and pH 6. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

***Penicillium roquefortii* at 0.95 a_w and pH 4.5**

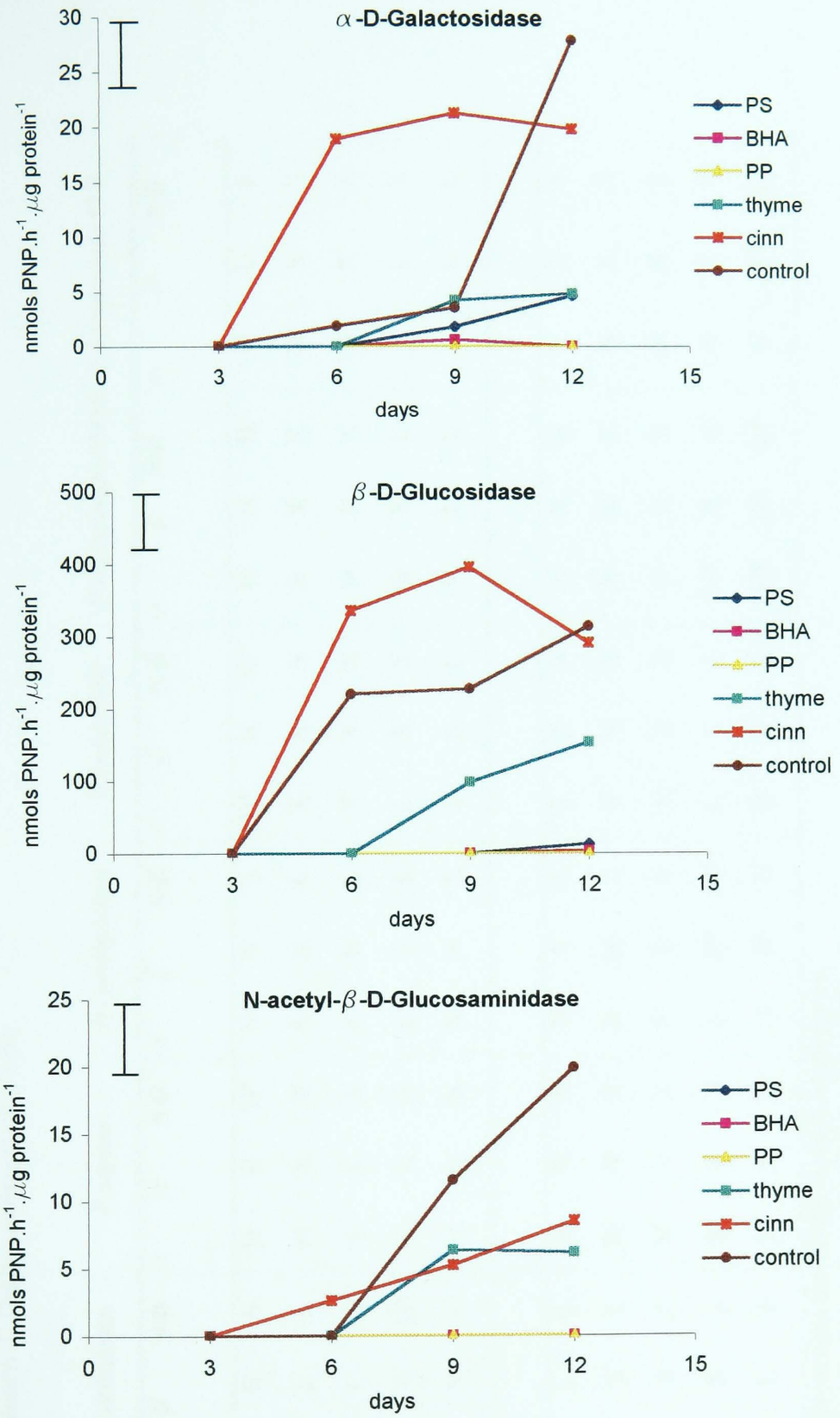


Figure 4.36 Effect of 150 ppm of potassium sorbate (PS), BHA, propyl paraben (PP) and thyme and cinnamon (cinn) essential oils on specific activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase from cultures of *Penicillium roquefortii* on 2% wheat flour agar, at 0.95 a_w and pH 4.5. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

Table 4.14 Effect of potassium sorbate (PS), Butylated hydroxyanisole (BHA), propyl paraben (PP) and thyme and cinnamon essential oils on enzyme production by bread spoilage moulds on wheat flour agar, 0.95 a_w and two pH levels (4.5 and 6). Key for enzymes: α-D-glucosidase (α), β-D-galactosidase (β) and N-acetyl-β-D-glucosaminidase (N-β).

Species	*A.ochraceus*			*E.repens*			*P.corylophilum*			*P.roquefortii*			*P.verrucosum M450*			*P.verrucosum PV3*		
ENZYME	α	β	N-β	α	β	N-β	α	β	N-β	α	β	N-β	α	β	N-β	α	β	N-β
pH 6																		
PS	N	H	N	H	H	H*	N	N	L	H*	N	H*	H	H	H	H	L	L
BHA	L	L	L	N	X	X	N	N	L	X	X	X	N	X	X	N	X	X
PP	L	L	L	N	L	X	N	N	L	X	X	X	N	X	X	N	X	X
THYME	H*	N	H*	N	L	X	L	L	L	L	X	X	N	X	L	N	L	X
CHNNAMON	L	N	L	N	L	X	N	N	L	L	N	L	N	L	L	N	L	L
pH 4.5																		
PS	L	L	L	N	N	X	N	X	X	X	X	X	N	X	X	N	X	X
BHA	L	X	L	N	N	X	N	H*	L	X	X	X	N	X	X	N	X	X
PP	L	X	L	N	N	X	N	L	L	X	X	X	N	X	X	N	X	X
THYME	H*	N	L	N	N	X	N	H*	H*	L	L	L	H*	X	X	N	X	X
CINNAMON	L	L	L	N	N	X	N	H	N	H	H	L	H*	H*	H*	N	H*	H*

L Lower activity than control for all days of incubation
H Higher activity than controls for all days of incubation
H* Higher activity than controls from day 9th of incubation
N No significant effect
X Enzyme activity completely inhibited

4.6.3 Comparison of enzyme production on 2% wheat flour agar and on bread analogues

The effect of substrate on the levels of fungal enzymatic activity was evaluated at the combination of environmental factors that better simulate the intrinsic characteristics of bread products, i.e. 0.97 a_w and pH 6. The species *A.ochraceus*, *E.repens* and *P.corylophilum* were used as a representation of the mycoflora. The combined effect of growth substrate and preservative type was also studied. Doses of 300 ppm of all compounds were used for comparison.

Figure 4.37 compares the levels of total and specific activity of α-D-galactosidase produced by *P.corylophilum*, *E.repens* and *A.ochraceus* on 2% WFA and bread analogue at 0.97 a_w and pH 6. Much higher enzyme production levels by spoilage fungi were observed on bread analogues than on WFA ($p<0.001$).

Both total and specific activities of all three enzymes increased significantly ($p<0.001$) with incubation time, with maximum levels generally observed after the 9-12 days incubation. Again, N-acetyl-β-D-glucosaminidase was overall the most actively produced enzyme.

Figure 4.38 shows the effect of 300 ppm of potassium sorbate, antioxidants and essential oils on total activity profiles of β-D-glucosidase by *P.corylophilum* on WFA and on bread analogues at 0.97a_w and pH 6.

The presence of preservative in the growth substrate exerted a statistically significant influence in all fungal enzyme activity. However, in contrast with WFA, in most cases the addition of all antifungal compounds resulted in a stimulation on enzymatic production when compared to the untreated samples. This stimulation in activity was significant particularly after 9-12 days of incubation ($p<0.001$). Only the effect of cinnamon oil appeared to be significant overall species and throughout the incubation period. Table 4.15 depicts for all enzymes and species, the preservatives that exerted a significant effect on total and specific enzyme production on bread analogues.

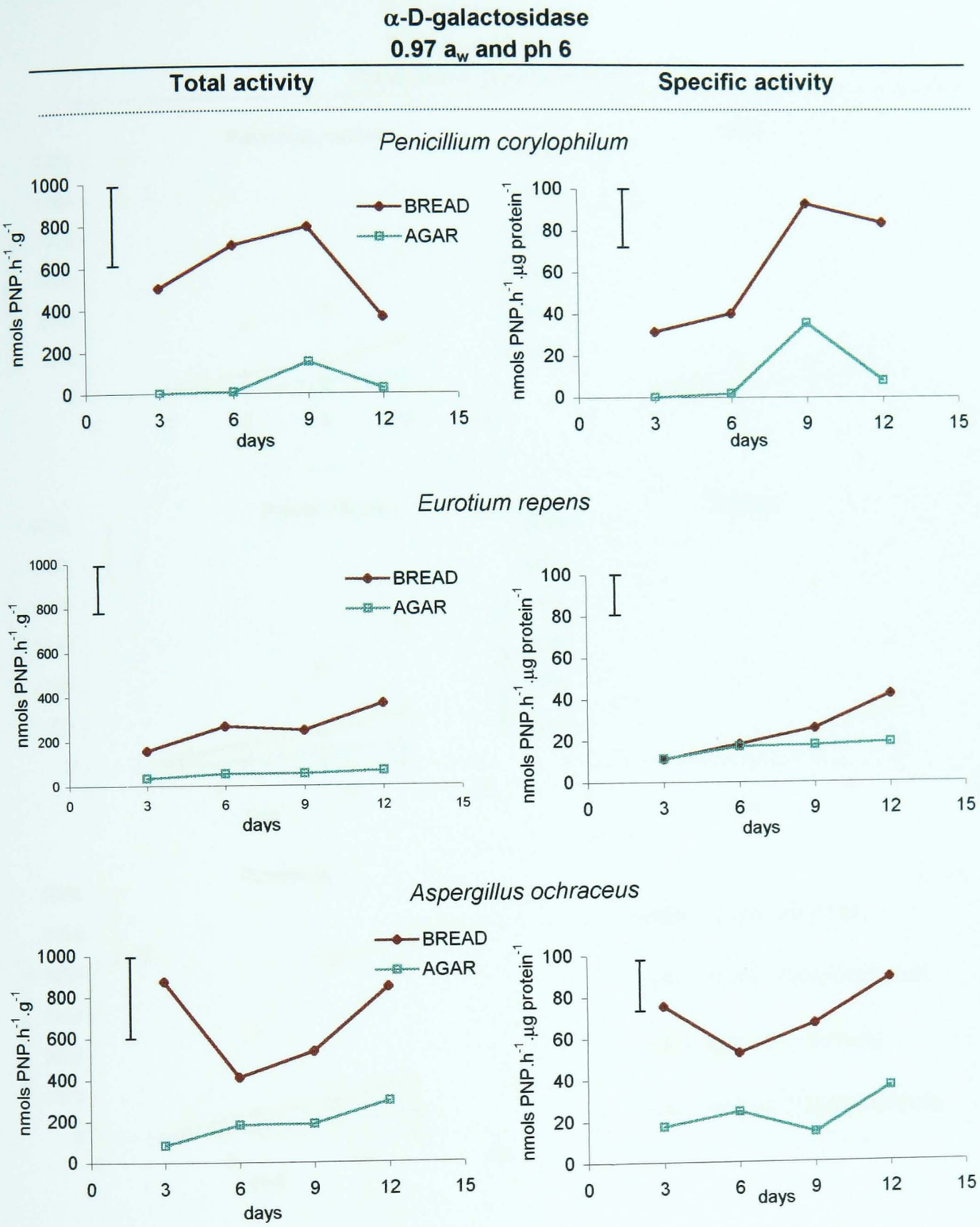


Figure 4.37 Effect of growth substrate (2% wheat flour agar versus bread analogues) on total and specific enzyme activities of α-D-galactosidase on cultures of *Penicillium corylophilum*, *Eurotium repens* and *Aspergillus ochraceus* at 0.97 a_w and pH 6. Bars indicate Least Significant differences (LSD) at $p < 0.05$.

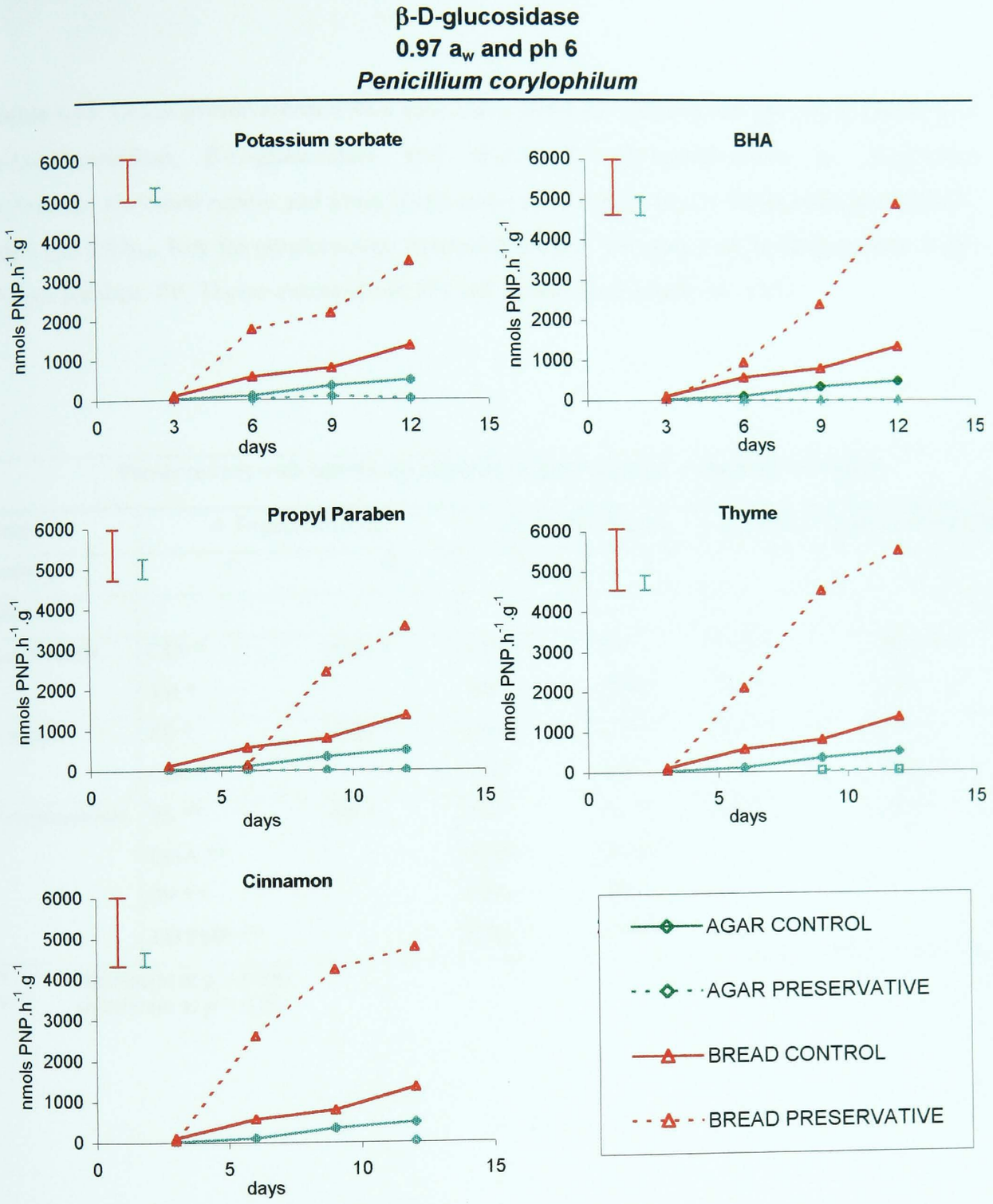


Figure 4.38 Effect of growth substrate and presence of different preservatives (at doses of 300 ppm) on total activity of β-D-glucosidase of cultures of *Penicillium corylophilum* at 0.97a_w and pH 6. 'Agar-control'/'bread-control' refers to cultures on WFA/bread with no preservative added and 'Agar-preservatives'/ 'bread-preservative' refers to cultures on WFA/bread with 300 ppm of preservative. Bars indicate Least significant Differences at $p < 0.05$.

Table 4.15 List of preservatives with a significant effect on total (T) and specific (S) activity of α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase *by Aspergillus ochraceus, Eurotium repens* and *Penicillium corylophilum* growing on bread analogues at 25°C, pH 6 and 0.97a_w. Key for preservatives: Potassium sorbate: PS, butylated hydroxyanisole: BHA, Propyl paraben: PP, Thyme essential oil: TH and cinnamon essential oil: CIN

Preservatives with significant effect on enzyme activity compared to controls						
Enzyme	α-D-galactosidase		β-D-glucosidase		N-acetyl-β-D-glucosaminidase	
Activity	T	S	T	S	T	S
Species						
A.ochraceus	CIN * TH *	CIN *	CIN * TH *	PS* CIN *	CIN * TH *	BHA ** TH*
E.repens	PS * CIN *	CIN *	CIN * TH *	CIN * TH *	CIN *	PS ** BHA **
P.corylophilum	PS ** BHA ** PP ** THYME **	PP *	PS * BHA * TH* CIN *	PS ** BHA ** TH * CIN *	TH *	TH**

* significant at $p < 0.001$
** significant at $p < 0.05$

4.7 SUMMARY OF RESULTS

In this section a summary of the major new and novel findings are highlighted:

A Effect of existing preservatives on growth of spoilage fungi on wheat flour-based media at different environmental conditions (Sections 4.1 and 4.2)

- In the absence of preservatives, conditions of low a_w (0.85-0.80), high pH (7.5) and low temperature (15°C) led to the longest mould free shelf-life and slower mould growth.
- At low pH (4.5) the actual permitted and used levels (3000 ppm) of GRAS preservatives potassium sorbate, calcium propionate and sodium benzoate were effective at controlling growth of bread spoilage moulds both on wheat flour agar and bread analogues.
- At pH 6 and 7.5 the efficacy of high doses of preservative was significantly reduced being practically ineffective at the maximum pH level tested.
- The efficacy of all preservatives increased as the water activity of the media was reduced.
- The use of sub-optimal concentration of preservatives can lead to growth stimulation, even at pH levels where the preservative is more effective.
- *Eurotium* and *Aspergillus* spp showed faster growth rates and shorter shelf life than *Penicillium* and *Cladosporium* spp over all conditions.
- Potassium sorbate was slightly more effective at controlling mould growth than calcium propionate and sodium benzoate.

B Effect of antioxidants and essential oils on growth of spoilage fungi (section 4.3)

- Over all environmental conditions, antioxidants butylatedhydroxyanisole (BHA) and propyl *p*-hydroxybenozate (propyl paraben, PP) and essential oils of thyme, clove, cinnamon and bay were effective *in vitro* antifungal agents completely inhibiting growth of all species at doses of 500 ppm for antioxidants and 1000 ppm for essential oils.

- The *in vitro* antifungal activity of antioxidants and essential oils was maintained at the two pH levels studied (4.5 and 6), making them potential alternative compounds to preserve a wide range of bakery products.
- As the a_w of the substrate was reduced, lower doses of both antioxidants and essential oils were needed to achieve the same level of antifungal activity.
- The addition of antioxidants and essential oils into the bread analogues as an additional ingredient greatly reduced their antifungal activity, with almost no effect at relatively high doses (1000 ppm).

C Effect of preservatives and environment on ochratoxin A (OTA) production by *A.ochraceus* and *P.verrucosum* (section 4.4)

- In the absence of preservatives, no OTA was produced on wheat flour agar by *A.ochraceus* and *P.verrucosum.*
- *In vitro* addition of sub-optimal concentration of potassium sorbate (150 and 300 ppm) stimulated OTA production by two of the three strains of *P.verrucosum* studied from the 28th day of incubation at 25°C, with levels of up to 1.5μg OTA. g agar^{-1}.
- No ochratoxin was detected in the presence of 150 ppm of antioxidants and essential oils.
- In contrast to mould growth, the capacity for OTA production was strongly affected by the type of substrate. On bread analogues, all strains of *P.verrucosum* produced up to 3.2μg OTA g bread^{-1} after only 7 days of incubation with maximum production of up to 75 μg. g bread^{-1} after 28-35 days.
- OTA production was favoured in conditions of low a_w (0.95-0.93) and high pH (6)
- High variability on toxin production was observed between the different strains of *P.verrucosum* and between experimental replications.
- In the presence of organic acids in bread analogues, no OTA was produced at the combination of environmental and preservation conditions that led to 100% of growth inhibition (i.e. pH 4.5 and doses of 3000 ppm, over all a_w levels).

- Under the rest of conditions, all strains of *P.verrucosum* were able to produce detectable levels of OTA after 28 days incubation. Generally OTA production on bread analogues was lowered by the presence of 300 and 3000 ppm. However, some significant stimulation was observed for strain M453 of *P.verrucosum* in the presence of 300 ppm of both organic acids at pH 6 and $0.95a_w$.
- Interestingly, although the addition of 1000 ppm of antioxidants and essential oils into bread analogues did not significantly affect mould growth, a marked reduction on OTA production was recorded.

D Effect of environment and sub-optimal concentration of potassium sorbate on carbon source utilisation profiles and niche overlap between bread spoilage fungi (section 4.5)

- The amount of carbon sources that a species is able to utilised both alone and in relation to other species varied significantly with a_w, pH, T and presence/absence of potassium sorbate.
- In the absence of preservative, bigger niche sizes were observed under conditions of high a_w and temperature, and low pH. A major proportion of carbohydrates than aminoacids and vitamins were generally utilised.
- Over all species, *P.verrucosum* , particularly strain PV3, appeared to be the species nutritionally most advantage, with generally bigger niche sizes and $NOI_{spp/PV3}$ ratios.
- At pH 5, the addition of 300 ppm of potassium sorbate to the media led to 70-100% reduction on niche sizes of all species. *P.verrucosum* PV3 was the least affected species and become nutritionally dominant over the other species.,
- At pH 6, only *E.repens* was the only species affected by the presence of preservative with a significant increase in its niche size.
- An increase in the nutritional competition between species was observed with the use of sub-optimal concentration of potassium sorbate at pH levels where the preservative is less effective.

D Impact of existing and alternative preservatives on hydrolytic enzyme activity of spoilage fungi (section 4.6)

- Enzymes α-D-Galactosidase, β-D-glucosidase and N-acetyl-β-D-Glucosaminidase proved to be good indicator of the mould activity on wheat flour-based substrates. The activity generally increased with the time of incubation.
- Different levels of activity of the three key hydrolytic enzymes were observed between the different fungal species studied. N-acetyl-β-D-glucosaminidase was the enzyme that showed the highest levels of activity over all treatments and for all species except *P.roquefortii*. In contrast, α-D-galactosidase was produced in the lowest amounts. The production of β-D-glucosidase was species dependent
- Production of N-acetyl-β-D-glucosaminidase and α-D-glucosidase was stimulated at pH 6. In contrast, β-D-glucosidase appeared to be more active at lower pH (4.5).
- The addition of organic acids, antioxidants and essential oils at sub-inhibitory concentrations affected the level of both total and specific activity of the three enzymes.
- Over all environmental conditions, greater reduction of all enzymatic activity was observed the presence of antioxidants compared to organic acids.
- Essential oils of thyme and cinnamon significantly reduced all enzymatic activity on *in vitro* cultures of *Eurotium* and *P.verrucosum* while for other, such as *P.roquefortii* production of α-D-Galactosidase and β-D-glucosidase was stimulated
- All enzymatic activity was higher on bread analogues than on wheat flour agar
- Interestingly, although the addition of antioxidants and essential oils to bread analogues did not significantly affected the in situ growth, enzyme production significantly increased.

Chapter 5

DISCUSSION

5.1 ENVIRONMENTAL FACTORS AND MOULD GROWTH

As demonstrated in many studies, environmental conditions such as water activity (a_w) pH or temperature have a significant effect on growth of spoilage moulds. The intrinsic characteristics of the product will determine the type and extent of mould colonisation. However, inadequate storage (e.g. high humidity or temperature) can create environmental conditions that foster mould growth favouring colonisation by a range of species. For this reason, the effect of different environmental factors on lag times and mould growth was studied for some of the most common fungi involved in bread/cake spoilage. It was found that in the absence of preservatives, water activity of the substrate (a_w) was the parameter that exerted the most influence on their growth. Many studies have been carried out on the effect of a_w on the growth of a wide range of fungal species. Results from these studies are, however, not always easy to compare as they were generally undertaken on a wide range of different nutritive media and carried out over a wide range of environmental conditions.

On both wheat flour agar and on bread analogues, all fungal species grew faster as the a_w was increased. In agreement with other reports (Mossel *et al.*, 1995; Rahman and Labuza, 1999) no species was able to grow at the lowest a_w tested, 0.80. This result on wheat flour-based substrates is similar to that reported previously for other *Aspergillus* and *Penicillium* species. Furthermore, results obtained for *A.ochraceus* are comparable with those of Ramos *et al.* (1998) who found a minimum a_w level of 0.81 a_w when the fungus was grown in a barley extract agar.

In contrast, although a minimum a_w level for growth of *E.repens* has been reported to be around 0.70 (Rahman and Labuza, 1999), no growth was observed when this species was grown at 0.80 a_w on a 2% wheat flour agar. Similarly, Abellana *et al.* (1999) reported 0.82 as the minimum a_w for the growth of three species of *Eurotium* grown on cake analogues.

With regards to the pH, growth of all species on 2% flour agar was faster as pH was lowered. Previous studies by Lopez–Malo *et al.* (1998) demonstrated at 0.98 a_w. higher growth rates of *A.ochraceus* on potato dextrose agar at pH 5.5 than at pH 3.

These results together with the data obtained in this project suggest an optimum pH for *A.ochraceus* of between 4.5 and 5.5, which can depend on the a_w level and/or on the type of substrate used.

In terms of temperature, all species grew slower at the lowest temperature studied (15°C). For example, at 15°C and for all a_w x pH combinations studied, the ochratoxigenic species *A.ochraceus*, grew at <1.3 mm day^{-1}, whereas at 25°C rates of up to 2.8 mm day^{-1} were observed. Similarly, Ramos *et al.* (1998) reported growth rates of <1.5 mm.day^{-1} for different strains of this species growing at 15°C on barley extract agar.

Discrepancies in the literature regarding the effects of a_w, pH and T levels upon growth of spoilage microorganisms may be due to factors such as the substrate used for fungal growth, or strain to strain variation. The effect of the type of substrate on mould growth has been previously reported by Madhastha *et al.* (1990) who found significant differences in the fungal biomass of *P.verrucosum* when the fungus was grown on different cereal (wheat and corn) and oilseed substrates (peanut, soybean, rapeseed). In their study they concluded that wheat was the substrate that best supported growth of this species.

Moreover, Fustier *et al.* (1998), studied the effect of substrate (PDA vs. yellow layer cakes) on colonisation and outgrowth of spoilage microorganisms. They demonstrated that, even with identical exposures to air cabinet contamination, the inoculation levels varied greatly as a function of the exposed medium. Suprisingly, growth was found to be faster on PDA than on cakes under the same environmental conditions (differences of approximately 1 mm day^{-1}).

In the present study, similar substrate effects to those reported by Fustier *et al.* (1998) were observed at 25°C, particularly for *Penicillium* isolates at high a_w and low pH levels (4.5). For instance, faster growth of *P.corylophilum* on wheat flour agar than on bread analogues was observed (differences of 1.6 mm day^{-1}). In contrast, for other species such as *Eurotium repens*, the growth was significantly faster on bread analogue with maximum variations of up to 4.6 mm day^{-1}.

5.2 EXISITING CHEMICAL PRESERVATIVES FOR CONTROL OF GROWTH OF SPOILAGE MOULDS

To control mould colonisation and outgrowth on bread and most bakery products, chemical preservatives such as potassium sorbate and calcium propionate are incorporated in the product formulation. Currently, doses of 1000-3000 ppm (w/w) are used in the bakery industry to control product and economic losses due to microbial contamination. The need to reduce concentrations of chemical preservatives in foodstuffs as a response to consumer demand for chemical-free, 'fresh' and mild-processed foods has increased significantly. However, a reduction in the dose of the preservatives used may not always be as effective.

The use of high concentrations (3000 ppm w/w) of the food grade preservatives potassium sorbate, calcium propionate and sodium benzoate, completely inhibited growth of all species at pH 4.5 on both wheat flour agar and bread analogues. Preservative effectiveness decreased when pH was increased to 6. At the highest pH level tested, a high concentration of preservatives generally failed to control mould growth. Mutasa *et al.* (1990) also reported that doses of 0.1% of potassium sorbate at pH 4.5 and doses of 0.2-0.3% at pH 5 exhibit good inhibition of several *Eurotium*, *Aspergillus* and *Penicillium* species on malt extract agar.

An important stimulation of mycelial growth was observed when weak acid preservatives were used at sub-optimal concentrations. The effect was particularly apparent for the ochratoxigenic species *A.ochraceus* and *P.verrucosum.* Similarly, growth stimulation of *Penicillium* and *Aspergillus* spp. was also found on tobacco extract media in the presence of higher doses of sorbates (0.1-0.4%) (Mutasa and Magan, 1990). In agreement with these authors, the effect was more evident when the fungus was grown on wheat flour agar than on bread analogues, probably due to the fact that in a poorer nutritional media, fungi may use the preservative molecules as a carbon source.

Stimulation of mould growth occurred generally at the higher pH levels, 6 and 7.5, where the preservative is less effective. This is related to the fact that at the lowest pH level studied (4.5), the percentage of the acid present in its undissociated, and thus 'active', form is higher (see Table 1.3 p. 18). In contrast, when the pH is increased to 7.5, the percentage of this undissociated form is almost zero, and therefore the salt is less effective at inhibiting spoilage fungi. Similar findings for *Eurotium* spp on wheat flour agar have been recently published by Marin *et al.* (2003). This pH-dependent activity of weak organic acids has also been previously reported by other authors (Garza *et al.*, 1993).

In some species, as some environmental conditions became unfavourable for mould growth (low a_w and temperature) stimulation was more apparent. Although *P.corylophilum*, for example, was inhibited on wheat flour agar at 25°C, pH 4.5 and 0.95 a_w, by 30 ppm of calcium propionate (9.7% inhibition), at 15°C with the same concentration of preservative, its growth was stimulated by up to 50% (data not shown).

On the other hand, when the fungi were grown under stress conditions (low a_w levels) the effectiveness of the preservative increased. An increase in efficacy of sorbates by a_w reduction has been previously reported (Liewen and Marth 1985; Thakur and Singh, 1994). Similar effects of environmental factors on the efficacy of existing preservatives were observed on bread analogues. Generally, growth inhibition was lower in media and conditions that better supported growth.

Over all conditions, potassium sorbate appeared to be more efficient at inhibiting mould growth than calcium propionate and sodium benzoate on both wheat flour agar and bread analogues. Similar findings were reported by Friend *et al.* (1995) on Mexican wheat tortillas.

From the data obtained in this study it may be suggested that sub-optimal concentrations of the existing preservatives can effectively control, under certain conditions of a_w and pH, the growth of some of the species responsible for bread spoilage. However, in a food product with a mixed mycoflora, the inhibition of some

species may lead to a stimulation in the growth of tolerant species, since their competition for nutrients has been eliminated (Liewen and Marth, 1985). Moreover, in bakery products with a higher pH (7-8) such as some Spanish cakes, the use of salts of organic acids may be useless even at the maximum permitted level of 3000 ppm.

The combined effect of environmental factors and preservatives is part of the new "hurdle technology" concept (Leistner, 1992: Leistner, 2000). Most recently, Marin *et al.* (2002) indicated that the combination of hurdle technology with predictive microbiology may be the most suitable tools to develop new and safer products.

With this in mind, some authors have investigated the synergistic effect of acidulants and organic acids in bread preservation. McNaughton *et al.* (1998) studied the combined effect of vinegar and calcium propionate on rope and mould shelf-life of brown South African bread. In their study they found mould shelf-life of 3.6 and 1.9 days when calcium propionate was used alone at concentrations of 0.3%. When calcium propionate was combined with equal amounts of vinegar (0.1 % of each) similar or even greater mould shelf-life was evident.

Although lowering the pH of the product would certainly increase the antifungal effectiveness of organic acids, other parameters of bread making could be adversely affected. Friend *et al.* (1995) for example, demonstrated that a small reduction in pH (from 5.8 to 5.5) in Mexican tortilla dough, preserved with 0.3% of calcium propionate or 0.3% of potassium sorbate, adversely affected dough machinability with increasing viscosity and decreasing elasticity of the final tortilla product.

5.3 ANTIOXIDANTS AND ESSENTIAL OILS FOR CONTROL GROWTH OF SPOILAGE MOULDS

Many studies are being carried out in order to identify alternative antifungal and antibacterial compounds that could replace or allow a reduction in the dose of existing preservatives used for food preservation. In the present study, plant essential oils and antioxidants were screened for antifungal activity and for their possible use in bread preservation.

(a) Antioxidants

Four phenolic antioxidants were studied: butylated hydroxyanisole (BHA), propyl *p*-hydroxybenzoate (propyl paraben), butylated hydroxytoluene (BHT) and propyl gallate (PG). BHA, BHT and PG were chosen because, although used in foods primarily to prevent the auto-oxidation of lipids, they have also been shown to possess antimicrobial activity against a wide range of microorganisms. On the other hand, propyl paraben is a derivative of benzoic acid with proven antimicrobial activity and lower sensitivity to changes in pH than its originating acid.

From all four antioxidants, BHA and PP showed the strongest *in vitro* antifungal activity, completely inhibiting all fungal growth at doses of 500 ppm. In contrast, BHT and PG exhibited poor activity with lag phases of 6-11 days and between 15-50% inhibition of growth at the maximum dose tested (1000 ppm). Furthermore, stimulation of growth of the ochratoxigenic species *P.verruosum* was observed on wheat flour agar in the presence of the lowest concentration (100-500 ppm) of BHT and PG.

Similarly, Thomson (1992) found no effect of 1000 ppm of BHT and PG against several *Penicillium* and *Fusarium* species on potato dextrose agar and potato dextrose broth over a period of time of 7 days. Jay (2000) also reviewed BHA as generally more inhibitory than BHT to bacteria and fungi, with the latter being more viristatic.

In the present study, the *in vitro* antifungal efficacy of BHA and propyl paraben appeared to be maintained at the different a_w and pH levels studied. In contrast, with existing preservatives, a relatively low effect of pH on the antifungal activity of antioxidants was found, which make them suitable candidates for the preservation of a wider range of bakery products. In all treatments, a dose of 200 ppm of either antioxidant was sufficient to completely inhibit growth of *Penicillium* and *Eurotium* isolates. *A.ochraceus* was able to grow at this concentration although slowly, and with lag times of 10 days (> 8-9 days more than the controls). In general, estimated MIC values of BHA and PP on wheat flour agar ranged between 200 and 500 ppm.

With regards to the type of antioxidant, BHA showed stronger antifungal activity than propyl paraben, especially at low concentrations (50-150 ppm). Previously, Thomson (1992) concluded that BHA was a more effective phenolic antioxidant than PP for inhibiting growth of several *Aspergillus*, *Penicillium* and *Fussarium* species on potato dextrose agar and reported MIC values ranging from 250-500 ppm. They also found higher MIC values for *Aspergillus* than for *Penicillium* and *Fusarium.* Khan *et al.* (1999) also reported BHA as the most promising *in vivo* control agent of crown rot disease of bananas in combination with low concentrations of benzoic acid and propyl paraben.

In contrast, in a later study, investigating the effect of 200 ppm of BHA and PP at different pH levels, against the same group of fungal strains as in their previous work, Thomson *et al.* (1993) found that PP was generally more effective in reducing conidial germination and mycelial growth. More recently, Torres *et al.* (2002) also concluded that PP was a more effective antioxidant at inhibiting growth of mycotoxigenic *Fusarium* spp on irradiated maize at high water availabilities (0.95-0.995) with a MIC of 500ppm.

However, similarly to that observed for existing preservatives, the use of very low concentrations of BHA and particularly PP (50 ppm) on wheat flour agar, resulted in enhancement of mould growth. Other authors have suggested the possible adverse effect of BHA at very low doses (10 ppm) on fungal toxin production (see Ray and

Bullerman, 1982), finding almost twice the amount of toxin produced in the presence of 10 ppm of BHA compared to the controls and with no effect on mycelial growth.

It is evident that BHA and PP exert an important antifungal activity in culture media that can vary with dose, fungal species/strain and type of substrate. The effect of substrate on the antifungal efficacy of antioxidants was very significant when these compounds were added to bread analogues. A much lower antifungal activity of BHA and PP on bread analogues was observed, compared to that found *in vitro*. No effect on lag times and growth rate of any species was observed with doses ≤ 300ppm. *Penicillium* and *Eurotium* isolates were only significantly inhibited with the highest dose of BHA and PP although < 30% inhibition of growth was recorded. It has already been suggested that, in general, higher concentrations (up to 50 times more) are required to inhibit mould growth in foods than it is in culture media, specially in food with a high-fat content (Jay, 2000).

(b) Essential oils

From the twenty different plant essential oils studied (see Table 2.4, p.49) only clove, thyme, bay and cinnamon completely inhibited growth of all fungal species on wheat flour agar at doses of 500 ppm and 0.95 a_w. In contrast, growth of the ochratoxigenic species *A.ochraceus* and *P.verrucosum* was stimulated to some extent by the presence of the other oils tested.

In combination with different environmental conditions (0.93-0.97 a_w and pH 4.5-6), oils of clove, thyme, cinnamon and bay, were effective at completely controlling the growth of spoilage fungi at doses of 1000 ppm (0.1%, w/w). Only *A.ochraceus* and *E.repens* grew with 500 ppm of some oils with lag times of 7-15 days. Regardless of a_w and pH, 70-100% inhibition of growth was generally found at doses of 500 ppm. In general, MIC values for the oils varied from 200 to 1000 ppm dependent on the type of oil used, species, a_w and pH levels. At 0.93 a_w for instance, MIC values of 100-200 were recorded at pH 6 and from 100-500 at a lower pH (4.5).

Although for some species, no statistically significant differences of activity between the different oils were recorded, growth of *A.ochraceus* and strain PV3 of *P.verrucosum* (PV3) appeared to be more effectively controlled with cinnamon and bay than with clove and thyme essential oil. Similarly, Patkar *et al.* (1993) found that 500 ppm of cinnamon oil completely inhibited growth of the aflatoxigenic species *A.flavus* on yeast extract broth and on agar media during 7 days incubation, whereas up to 1250 ppm of clove essential oil was necessary to exert the same inhibitory activity. Azzouz and Bullerman (1984) also reported a high anti-mould activity of clove and cinnamon oil against several *Aspergillus* and *Pencillium* species.

Although many other studies depict the strong effect essential oils have on mould growth, results have not always been similar. For instance, in the present study, 500 ppm of thyme essential oil completely inhibited growth of *A. ochraceus* on 2% flour agar over 30 days. However, Paster *et al.* (1990) reported growth of the same species, with colonies of up to 35 mm diameter on PDA and in the presence of the same concentration of thyme essential oil. Furthermore, Özcan (1998) found growth of *Aspergillus parasiticus* on CZ agar supplemented with of 1% thyme (wild and black) oil extract.

These variations may be attributed to inter-species variation in sensitivity to a specific essential oil, or to the fact that, the chemical composition of the essential oil can vary with the plant origin and extraction method. In fact, while in the current study thyme oil was a commercial essential oil obtained from Spanish varieties, Paster *et al.* (1990) extracted the oil themselves from leaves of Israeli plants. In addition, Lis-Balchin *et al.* (1998) demonstrated the importance of chemical composition and origin of commercial essential oils in their *in vitro* antibacterial and antifungal efficacy. For instance, while up to 79% of growth inhibition (measure as mycelial weight) of *A.ochraceus* was achieved with French marjoram essential oil, only 8% inhibition was found when Spanish marjoram oil was used.

Supporting present findings, recent studies demonstrated the *in vitro* effectiveness of clove essential oil against the phytopathogenic fungi *Alternaria alternata* and *Fusarium chlamydosporum* (Zafar and Iqbal, 2002). They reported clove activity as

fungistatic with minimum inhibitory concentrations of 0.05% (500 ppm). Above this concentration, lysis of conidia and inhibition of mycelial growth were detected. Similarly, Zhou *et al.* (2000) established MIC of clove oil against several *Aspergilllus* and *Penicillium* spp, in nutritive media, at 400 ppm (v/v). Interestingly, they also found that activity of the oil could be doubled when emulsified with glycerine, lowering the MIC to 200 ppm.

In contrast to what was observed on wheat flour agar, Juglal *et al.* (2002) found that clove was more effective than cinnamon oil against growth of *A.parasiticus* and *F.moniliforme* in broth and in corn patty cultures. They suggested that overall, spice essential oils can successfully inhibit mould growth, regulate production of fumonisins and prevent formation of aflatoxins in corn. Similar conclusions were drawn previously by Patkar *et al.* (1994) for storage of rice.

However, as with phenolic antioxidants, an important reduction of antifungal activity was observed when oils of thyme, clove, cinnamon and bay were added to bread analogues. Oil concentrations of 1000 ppm on bread analogues had little effect on mould growth. A statistically significant effect was only observed for *E.repens* at doses $\geq$ 500 ppm with maximum growth reductions of about 27-52%.

This decrease in activity of essential oils and antioxidants when added to bread analogues may be due to the composition of the food, which contains fats and proteins which can immobilise and inactivate important antifungal components of the oil. Due to their low solubility in water and high lipophilicity, it is possible that essential oils and antioxidants may migrate out of the aqueous phase into the oil phase thus effectively not being able to enter, by osmosis, the fungal cell and ultimately allowing the mould to grow. However, it could be argued that this lipid affinity would benefit the biological activity of such compounds since higher partition coefficients may be expected. Further investigation will be important to examine the causes of these reductions in activity in food matrixes.

Nevertheless, although essential oils may not be as effective as initially expected when used as an ingredient in processed foods such as bread, the volatile components

represent a promising means of preserving the microbiological quality of stored grain and as part of active packaging of foods. Seok *et al.* (2000) for example, successfully incorporated clove oil in low-density polyethylene films. With oil-treated films they observed an increase in antimicrobial effects against *Fusarium oxysporum* compared to control films but with no effect on the physical properties of the films. In addition, Nielsen and Rios (2000) studied the effect of several essential oils as an atmospheric component in active packaging. They demonstrated a high fungistatic effect with 1µl of cinnamon and mustard oils against several bread spoilage moulds with complete inhibition of growth over a period of 14 days.

In general, essential oils with aldehydes (cinnamaldehyde from cinnamon) and phenols (eugenol and carvacarol from thyme, clove and cinnamon) as their main constituents appear to be the most promising natural products for food preservation. Similarly, Lis-Balchin *et al.* (1998), after studying the antimicrobial activity of 53 different essential oils concluded that oils with consistent biological activity against both bacteria and fungi, were those containing primarily cinnamaldehyde, eugenol and citral. A GC-MS analysis of the commercial oils used in this project found that eugenol was the main component of cinnamon and clove oils, thymol of thyme and chavicol and euginol of bay essential oil (data kindly provided by Prof. P.V. Nielsen, DTU, Denmark).

Apart from the biological activity, other important aspects such as regulations need to be taken into consideration when studying new alternative compounds for food preservation. In the case of antioxidants, although the *in vitro* MIC values are ten times lower than those of weak organic acids, they are also very close to the actual maximum levels permitted in most foods (200-300 ppm: 89/107/EEC, 95/2/EC and 97/77/EC). These limits are related to their specific use as antioxidants, and as for essential oils, no regulation has been drawn to date for their use as antimicrobials.

5.4 OCHRATOXIN A (OTA) PRODUCTION BY *A.OCHRACEUS* AND *P.VERRUCOSUM*

In addition to the economic losses caused by visible growth of spoilage moulds on bread, health hazards associated with the presence of mycotoxins is also of concern. Ochratoxin A (OTA) is a mycotoxin commonly associated with bread products. Several authors have indicated bread as one of the main sources of OTA daily intake (Legarda & Burdaspal, 2001; Cholmakov *et al.*, 2000; Gareis *et al.*, 2000). The presence of OTA in bread mainly comes from the wheat flour used for its manufacture, since its presence in wheat grain or in flour is only partly destroyed during the breadmaking process (Subirade, 1996).

The *in situ* formation of OTA on mouldy bread is believed to be a risk for human health either directly, as a result of people eating mouldy bread or indirectly, as a result of people consuming the products of animals fed with mouldy bread (Osborne, 1980; Legan, 1993). Although the likelihood of people eating spoiled bread is very low in developed countries, the incidence in developing countries can be greater. For instance, a close statistical association has been reported between cirrhosis of the liver and consumption of mouldy steamed bread in China (Brun *et al,* 1989). Surprisingly, very little information is available on the impact of preservatives on the potential for OTA production on bread by spoilage moulds.

For this reason, the potential for OTA production of one ochratoxigenic strain of *A.ochraceus* and three of *P.verrucosum* (M450, M453 and PV3) on wheat flour agar and on bread analogues was evaluated. Since different bread products have different a_w and pH levels, the influence of such factors on OTA production was also considered. Moreover, the study of different a_w levels also simulated possible changes in humidity that can occur during storage of wrapped and unwrapped bread.

On wheat flour agar at 25°C and at a_w levels from 0.93-0.97, it was found that no OTA was produced by either species during an incubation period of 54 days. Under the same environmental conditions, on bread analogues, up to 3.2 $\mu g\ g^{-1}$ were

produced by all strains of *P.verrucosum* after only 7 days incubation at 25°C with a maximum production after 28 or 35 days.

Although little differences in growth rates of *P.verrucosum* between wheat flour agar and bread analogues were generally observed (< 1 mm day^{-1}), the substrate influence on ochratoxin A production was much greater. Similarly, Madhyasta *et al.* (1990,1993) reported significant variations in OTA production by *P.verrucosum* when the fungus was grown on wheat than on barley, the former being the most favourable. Luchese and Harrigan (1993) also confirmed that the nutrient supply strongly affects secondary metabolism of fungal cultures including mycotoxin production.

In contrast to the high levels of OTA produced by *P.verrucosum*, the ochratoxigenic strain of *A.ochraceus* failed to produce toxin in either type of substrate. Some reviews report that, even in inoculated bread, not all mycotoxigenic moulds are able to produce mycotoxins (Legan, 1993). This variation also appears to occur in naturally contaminated breads. Nevertheless, because the incidence of *Penicillium* spp. in spoilage of white bread, in temperate climates like the U.K., is generally greater than *Aspergillus* spp, more attention was given to the potential for OTA production by the former species.

Contrary to that observed for growth, OTA production by *P.verrucosum* was favoured by a_w levels of 0.95-0.93 for all strains and pH levels (4.5 and 6). Although optimum a_w levels for OTA production have been reported at ≥ 0.97 a_w (Moss, 1996; Ramakrishna *et al.*, 1996), other authors observed interesting intraspecific variations, with some strains of *P.verrucosum* producing maximum levels of OTA at 0.95 a_w while for others, under the same conditions, 0.99 a_w was optimum (Northolt *et al.*, 1979). Patterson & Damoglow (1986) studied the effect of a_w (0.75 to 0.95) and pH (4 to 8) on OTA production by *A.ochraceus* and *P.verrucosum* on bread analogues over a period of 10 days. Similar to our findings, they reported 0.92 a_w as the optimum for OTA production.

In the current research, strain PV3 produced significantly higher levels of OTA than the other two strains particularly at 0.93 a_w and pH 6. These results support the idea of

a high strain-dependence on the potential for OTA production. The influence of pH on toxin production also appeared dependent upon the strain and a_w. At 0.93 a_w, strain PV3 produced much higher levels of ochratoxin A at pH 6 than 4.5 in agreement with other reports (Patterson & Damoglou, 1986; Moss, 1996; Aziz and Moussa, 1997).

The addition of existing preservatives to wheat flour agar and to bread analogues, at both high and sub-optimal doses (3000 ppm and 300 ppm), had a significant effect on toxin production. Moreover, although no OTA was detected *in vitro* in the absence of preservatives, contents up to 1.5 $\mu g\ g^{-1}$ were produced by strain PV3 of *P.verrucosum* when sub-optimal concentrations of potassium sorbate (150 and 300 ppm) were used, at pH 6 and 0.97 a_w, after 35 days.

On bread analogues the preservative effect on OTA production was more evident. No OTA was detected on bread analogues at treatments that led to 100% growth inhibition, i.e. at pH 4.5 and high concentrations of preservative (3000 ppm). Similarly, Michail *et al.* (1994) found complete *in vitro* inhibition of aflatoxin production by *A.flavus* at doses of 1.5% potassium sorbate over 14 days. Although generally, lower OTA concentrations were observed when either potassium sorbate or calcium propionate was added to bread analogues at doses of 300 and 3000 ppm, some stimulation of OTA production by strain M453 was found in the presence of sub-optimal doses (300 ppm) at pH 6 and 0.95 a_w. In any case, the increase in OTA production was not related to an enhancement of mould growth.

It is noteworthy that the preservative effects presented in this study are related to one time of incubation. As some authors have observed (Garza *et al.*, 1993), it may well occur that, once initial inhibition is over, mycotoxin production can reach similar levels compared to those obtained under preservative-free cultures.

Interestingly, and contrary to what was found for mould growth, the addition of antioxidants and essential oils into bread analogues, at doses of 1000 ppm, significantly reduced OTA production of two of the strains of *P.verrucosum* studied, particularly in the presence of thyme essential oil. Nevertheless, the inhibition of OTA

production by *in situ* addition of 1000 ppm of antioxidants and essential oils was lower or similar to that observed with 300 ppm of potassium sorbate.

Other studies have demonstrated the inhibitory effect of plant essential oils against mycotoxigenic spoilage fungi and mycotoxin production. However, this inhibition was normally correlated with a reduction on mycelial growth (Bilgrami et al., 1992; Patkar et al, 1993; Mahmoud, 1994; Basilico and Basilico, 1999).

5.5 CARBON UTILISATION PROFILES AND NICHE OVERLAP BETWEEN SPOILAGE FUNGI IN THE PRESENCE OF SUB-OPTIMAL CONCENTRATIONS OF POTASSIUM SORBATE.

Most research studies carried out on control of mould spoilage on food products focus the study on the effect that preservation hurdles have on single fungal species. It is important to consider that fungal contamination on a food surface is not only due to one species but by a group of species that interact and compete for nutrients. Carbon catabolism is the major source of energy that fungi need for growth, providing at the same time carbon sources for cellular synthesis.

The number of carbon sources that a species is able to catabolize compared to that of its surrounding species, gives information about its relative ability to compete in a particular niche. In this study, interaction of spoilage species was considered from a nutritional point of view. However, conclusions drawn from the nutritional niche overlap indices presented are only indicative of the fungal dynamics on the food product, since other factors such as environment or production of secondary metabolites may contribute to a species dominance within a fungal population.

Other authors have studied the effect of environmental conditions on niche overlap index of fungal species. However, no previous studies have included preservatives as an additional factor.

Similar to what was reported by Lee and Magan (1999) and Marin *et al.* (1998b), niche sizes of all species decreased as the a_w of the substrate was reduced. The minimum a_w level for carbon catabolism was influenced by temperature and the presence of preservative. At both pH levels *E.repens,* for instance, required higher a_w levels for growth initiation when the temperature was reduced from 25 to 15°C. This supports results published by Abellana *et al.* (1999). These authors studied the effect of a_w and temperature on growth of different *Eurotium* species on sponge cake analogues and concluded that at the highest temperatures tested (25-30°C) the range of a_w conditions for growth was wider than at lower temperatures.

Although growth on minimal media occurred at the lowest a_w level tested, this was mainly mycelial growth. In fact, sporulation was only observed at higher a_w levels (0.93-0.97). This also occurred when the fungi were grown on wheat flour agar. Previously, Beuchat (1983) indicated that generally, higher a_w is required for spore formation than germination. This suggests that nutritional requirements for mycelial growth are lower to those for sporulation. This is important, particularly from the point of view of food spoilage, because outgrowth of germinated spores is often used to determine the shelf-life of the product.

For treatments of 0.97 a_w, pH 5 and 25°C all species utilised to a higher number of CS. The ochratoxigenic *P.verrucosum* was found to be the species with the highest overall niche size. Under this condition, *P.verrucosum* utilised more than 93% of the CS used by the rest of the species ($NOI_{spp/PV3} < 0.93$) sharing less than 87% of its niche size with the other test species, giving *P.verrucosum* nutritional advantage. This predominance may be attributed to the ability of this species to utilise more complex carbon sources such as thiamine, DL-pantotheic acid or folic acid that most of the other species were unable to catabolize. As the a_w and the temperature were reduced, *P.verrucosum* and other *Penicillium* spp became less competitive. At lower temperature, more species occupied different nutritional niches, which may indicate a higher risk for their colonisation of bread since competition for the same nutrients is reduced.

The presence in the medium of potassium sorbate affected both niche sizes and niche overlap index. At pH 5, *P.verrucosum* PV3 was the species least affected by the addition of potassium sorbate and therefore appeared to be nutritionally dominant over the other species. Ordaz-Ortiz and Vazquez-Carrillo (1997) demonstrated an increase in the natural occurrence of *Penicillium* spp on Mexican corn when 0.1% of weak-acid preservatives such as potassium sorbate and calcium propionate were added into the product, compared to untreated samples where *Aspergillus* and *Cladosporium* species were also present.

At higher pH levels (pH 6), the addition of 300 ppm of potassium sorbate increased the inter-specific competitions for nutrients with more coexistence between species particularly *A.ochraceus* which shared more carbon sources with *P.verrucosum* and *P.roquefortii* when potassium sorbate was present. The increase in competition for nutrients with *P.verrucosum* observed may have an effect on toxin production. Stress conditions (reduction on the availability of nutrients) may lead to changes in secondary metabolites such as OTA. The fact that in naturally contaminated bread, lower concentrations of OTA are generally detected compared to experimentally inoculated bread, could be attributed to competition of other species. Ramakrishna *et al.*, (1996), for example, found that OTA production by *P.verrucosum* on barley grain decreased at 20 °C and 0.97-0.95 a_w by competition with *A.flavus.*

The use of potassium sorbate at conditions where its effectiveness is diminished can therefore adversely affect the microbiological quality of the product perhaps by promoting growth and toxin production of ochratoxigenic species such as *P.verrucosum,* or by increasing the incidence of fast growing species such as *Eurotium* spp resulting in shorter product shelf-life.

5.6 IMPACT OF EXISTING AND ALTERNATIVE PRESERVATIVES ON HYDROLYTIC ENZYME ACTIVITY OF SPOILAGE FUNGI

As described in the previous section, fungi are heterotrophic organisms, i.e. they need pre-formed organic compounds as energy sources and as carbon sources for cellular synthesis. In general, fungi can only absorb small soluble nutrients, such as monosaccharides and aminoacids, or peptides composed of two or three amino acids. Slightly bigger molecules, even disaccharides, need to be degraded before they are taken up into the cell, thus nutrition, at least for most fungi, is strongly dependent on the release of degradative enzymes (Deacon, 1997).

In this study, production of hydrolytic enzymes by spoilage fungi has been considered as a possible indicator of mechanisms of action of different types of preservatives. Hydrolytic enzymes are synthesised inside the fungal cell and when necessary excreted to the medium through the cell wall. Cell wall integrity is important for the survival of the fungal cell. Most antifungal compounds are believed to act by disrupting the cell wall, and thus increasing membrane permeability and ion flux. This has been recently reported as a possible mode of action of oregano essential oil and their two major constituents, thymol and carvacarol (Lambert *et al.*, 2001).

On the other hand, Conner and Beuchat (1984) suggested that the antimicrobial activity of several essential oils may be due to their effect on enzymatic systems, including those involved with energy production and synthesis of structural compounds. Similarly, Lopez-Malo *et al.*, (1998) inferred that phenolic compounds commonly found as part of essential oils such as vanillin can denature enzymes and interfere with the amino acids involved in spore germination.

The production of seven hydrolytic enzymes by spoilage fungi was studied on wheat flour agar (see section 3.9.3), but only three were detected: α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase. Similarly, other authors (Jain & Lacey, 1991 and Marin *et al.*, 1998b) also found higher levels of these enzymes produced by *Fusarium*, *Penicillium* and *Aspergillus* species colonising maize grain.

More recently, Keshri *et al.*(2002), reported that quantitative changes in these three enzymes occurred in bread substrates due to fungal colonization.

On wheat flour agar and for all species tested, high levels of N-acetyl-β-D-glucosaminidase were produced. In *Pencillium* isolates similar levels of β-D-glucosidase were produced. Overall, low amounts of α-D-glucosaminidase were detected. *Eurotium* spp. produced the least amount of all enzymes which suggests, together with results from the nutritional niche studies, that this species has relatively low nutritional demands for growth and product colonisation. This could explain the ability that *Eurotium* spp has for growth on a wide range of food matrices.

The effect of environmental conditions on enzyme production followed similar patterns as for growth. However, although some authors have found a correlation between fungal growth and hydrolytic enzyme production on maize (Marin *et al.*, 1998a), in this study no direct comparison of the two parameters was studied.

The presence of preservatives had a significant effect on the total amount of enzymes produced. Overall, α-D-galactosidase was less affected by the presence of preservatives as β-D-glucosidase and N-acetyl-β-D-glucosaminidase. It is possible that the levels of N-acetyl-β-D-glucosaminidase and β-D-glucosidase that a growing fungus produces can be related to the extent of the cell wall formation. The hyphal wall of most filamentous fungi consists of an inner primary wall composed of chitin microfibrils (a polymer of N-acetylglucosamine) and an outer wall composed of β1-3 and β1-6 glucans (polymers of glucose). These polymers are believed to be synthesised and broken down continuously during hyphal growth (Robson, 1999) and thus it may be inferred, that the production of N-acetyl-β-D-glucosaminidase and β-glucosidase will be indirectly related to the amount of chitin and β-glucans present and therefore the level of cell wall degradation .

It has been observed that the levels of N.-acetyl-β-D-glucosaminidase generally decreased to almost negligible levels in the presence of inhibitory concentrations of preservatives. This effect is more apparent when comparing the effect of potassium

sorbate at the two pH levels studied. At pH 4.5, when the preservative is known to be more effective, the levels of N-acetyl-β-D-glucosaminidase detected were very low. In contrast, at pH 6 the levels of the enzyme produced in the presence of preservative were generally higher or similar to untreated samples.

The antioxidants, butylated hydroxyanisole and propyl paraben exerted a stronger inhibitory effect on the production of the three enzymes. The addition of thyme and cinnamon essential oil resulted in a significant reduction of all enzyme production in some species such as *E.repens* and *P.verrucosum*, while for others like *P.roquefortii* production of α-D-galactosidase and β-D-glucosidase was stimulated.

On the other hand, all hydrolytic enzymes were produced at significantly higher levels when the fungi were grown on bread analogues. This was expected since bread analogues generally support growth better and represent a more nutritive substrate for the growing fungi.

Interestingly, although the addition of preservatives to the media did not appear to have any effect on mould growth, enzyme production significantly increased, particularly of N-acetyl-β-D-glucosaminidase. This might suggest that, although no effect on visible measured growth occurred, the presence of preservatives have an effect at a biochemical level and that moulds were able to overcome such hurdles.

It was difficult to draw general conclusions on the effect of existing and alternative preservatives on hydrolytic enzyme production. Important variations in the production of these enzymes were observed between treatments and species which were not always easy to related to growth differences. If, as suggested, preservatives at inhibitory concentrations perturb the cell wall and membrane, it would be interesting to study the direct effect of such preservatives on cell wall formation by perhaps directing the study on production of more related enzymes such as β-glucan and β-chitin synthases.

Chapter 6

CONCLUSIONS

AND

RECOMMENDATIONS FOR FURTHER WORK

6.1 CONCLUSIONS

From the experimental work carried out in this project the following conclusions can be drawn:

1 The use of actual permitted and used levels (3000 ppm) of weak-acid preservatives such as potassium sorbate, calcium propionate and sodium benzoate are effective at controlling growth of common spoilage moulds in bakery products characterised by low a_w and pH. Even at the maximum concentration, preservative effectiveness is strongly influenced by the pH of the food, being practically ineffective in products of high pH (near 7) as in the case of some Spanish cakes.

2 Over a wide range of environmental conditions, decimal reduction of the dose of existing preservatives resulted in a stimulation of the growth of spoilage moulds with product shelf-life being unaffected or even reduced.

3 Environmental conditions and sub-optimal concentrations of potassium sorbate affect the nutritional competition between spoilage moulds and may benefit growth and toxin production of ochratoxigenic species like *Penicillium verrucosum*. The higher incidence of *Penicillium* spp that naturally occurs on bread products may be explained by their nutritional advantage.

4 Ochratoxin A (OTA) production by *Penicillium verrucosum* is influenced by substrate, environment and by the presence of weak acid preservatives. If growth is allowed, all strains are capable of producing OTA on bread even with high concentrations of preservatives.

5 There is a need for alternative, more 'natural' compounds with stronger antifungal activity, with less dependence on environmental conditions.

6 The antioxidants, butylated hydroxyanisole and propyl paraben and essential oils of clove, thyme, cinnamon and bay, show good *in vitro* antifungal activity against a wide range of mould species with minimum inhibitory concentrations (MIC) ranging from 200-500 ppm for antioxidants and 200-1000 ppm for plant essential oils. Moreover, their biological activity appear to be low pH-dependent.

7 The addition of antioxidants and essential oils as an additional ingredient of bread reduces their antifungal efficacy probably due to the active molecules binding to food components, particularly lipids. Alternative modes of application, and their effect on product shelf-life are areas of further research to be investigated.

8 OTA production by *P.verrucosum* is inhibited more by the addition of antioxidants and essential oils into bread than its growth.

9 Spoilage moulds produce detectable levels of β-D-galactosidase, α-D-glucosidase and N-acetyl-β-D-glucosaminidase when growing on wheat flour-based substrates, that vary with changes in the substrate pH and a_w, and by the addition of existing and alternative preservatives. However, over all conditions and species, no general patterns on the effect of preservatives on hydrolytic enzyme production were observed. N-actyl-β-D-glucosaminidase may be a possible indicator of cell wall degradation caused by the presence of preservatives although its relationship should be further investigated.

6.2 RECOMENDATIONS FOR FURTHER WORK

Based on the research completed in this project, a number of key topics have been identified as being further opportunities for study. These are:

1 Investigate the use of essential oils and other antifungal compounds as part of modified atmospheres in active packaging of bakery products or as part of the packaging material in slow releasing systems that could allow a continuous supply of mould growth inhibitors.

2 Study the effect of essential oils on the incidence of different fungal species in situations of mixed mycoflora contamination and the impact of species interactions on mycotoxin production.

3 Investigate in more depth the cellular mechanisms involved in antifungal effects of different types of preservatives with particular attention to enzymes involved in the synthesis of the fungal cell wall and to Ca^{2+}-K^{+} cell pumps.

Chapter 7

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APPENDIX I

STATISTICS

I-1 EFFECT OF ENVIRONMENTAL CONDITIONS ON GROWTH OF SPOILAGE MOULDS ON 2% WHEAT FLOUR AGAR

Table I-1 Analysis of variance on the effect of water activity (a_w), pH, temperature (T), species (spp) and their interactions on lag times and growth rates on 2% wheat flour agar.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***df***	**MS**	***F***	**MS**	***F***
Spp	5	232.76	56.78 *	2.2246	127.12 *
T	1	479.94	117.07 *	2.55102	145.78 *
pH	2	29.86	7.28 *	0.40204	22.97 *
a_w	3	489578	1194.24 *	10.54143	602.38 *
Spp x T	5	74.45	18.16 *	0.32416	18.52 *
Spp x pH	10	6.07	1.48^{n}	0.05177	2.96 **
Spp x a_w	15	123.97	30.24 *	0.4095	23.40 *
T x pH	2	0.94	0.23^{n}	0.04761	2.72^{n}
T x a_w	3	81.63	19.91*	0.80678	46.10 *
pH x a_w	6	5.52	1.32^{n}	0.09511	5.44 *
Spp x T x pH	10	6.55	1.6^{n}	0.013413	0.77^{n}
Spp x T x a_w	15	36.28	8.85 *	0.05892	3.37 *
T x pH x a_w	6	2.61	0.64^{n}	0.02887	1.65^{n}

* significant at $p < 0.001$
** significant at $p < 0.05$
^{n} no significant $p > 0.05$

I-2 EFFECT OF EXISTING PRESERVATIVES IN COMBINATION WITH ENVIRONMENTAL FACTORS ON GROWTH OF SPOILAGE MOULDS ON 2% WHEAT FLOUR AGAR

Table I-2 Analysis of variance on the effect of water activity (a_w), pH, temperature (T), type of organic acid (pres) and dose, species (spp) and their two way interactions on lag times and growth rates of on 2% wheat flour agar.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***df***	**MS**	***F***	**MS**	***F***
spp	5	1169.6	43.7 *	3.1705	74.26 *
a_w	3	22157.2	828.88 *	36.599	857.28 *
pH	2	1461.8	54.68 *	0.7316	17.14 *
T	1	1541.6	57.67 *	5.3515	125.35 *
pres	2	749.1	28.02 *	0.3542	8.3 *
dose	3	3544.1	132.58 *	5.9367	139.06*
spp x a_w	15	487.8	18.25 *	1.7522	41.04 *
spp x pH	10	54.4	2.04 **	0.1102	2.58 **
spp x T	5	293.3	10.97 *	1.0596	24.82 *
spp x press	10	89.0	3.33 *	0.1541	3.61 *
spp x dose	15	106.5	3.98 *	0.4724	11.07*
a_w x pH	6	215.9	8.07 *	0.1872	4.38 *
a_w x T	3	421.8	15.78 *	2.4540	57.48 *
a_w x pres	6	94.4	3.53 **	0.0656	1.54 *
PH x T	2	12.1	0.45 ^{n}	0.236	0.55 ^{n}
pH x pres	4	40.8	1.53 ^{n}	0.0290	0.68 ^{n}
pH x dose	6	979.3	36.63 *	1.2101	28.35 *
T x pres	2	16.8	0.63 ^{n}	0.0325	0.76 ^{n}
T x dose	3	147.8	5.53 *	0.8837	20.7 *
pres x dose	6	187.9	7.03 *	0.0849	1.99 **

* significant at $p < 0.001$
** significant at $p < 0.05$
^{n} no significant at $p > 0.05$

I-3 EFFECT OF ENVIRONMENTAL CONDITIONS ON GROWTH OF SPOILAGE MOULDS ON BREAD ANALOGUES

Table I-3 Analysis of variance on the effect of water activity (a_w), pH and species (spp) and their interactions on lag times prior to grow and growth rates on bread analogues.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***Df***	**MS**	***F***	**MS**	***F***
Spp	7	32.196	33.8 *	11.6286	66.54 *
a_w	2	5.370	5.82 **	3.3929	19.41 *
PH	1	27.210	29.49 *	5.9151	33.84 *
Spp x a_w	14	2.969	3.22 **	0.3496	2.00^{n}
Spp x pH	7	10.763	11.66 *	1.7627	10.09 *
pH x a_w	2	4.848	5.25 **	0.3926	2.25^{n}

* significant at $p < 0.001$
** significant at $p < 0.05$
^{n} no significant at $p > 0.05$

I-4 EFFECT OF EXISTING PRESERVATIVES IN COMBINATION WITH ENVIRONMENTAL FACTORS ON MOULD GROWTH IN BREAD ANALOGUES

Table I-4 Analysis of variance on the effect of preservative type (pres) dose, water activity (a_w), pH and species (spp) and their two and three way interactions on lag times prior to grow and growth rates on bread analogues.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***df***	**MS**	***F***	**MS**	***F***
a_w	2	146.91	5.17 **	9.9294	73.08 *
pH	1	4623.85	162.78 *	52.105	383.49 *
pres	1	0.1	0 ^{n}	0.0684	0.5 ^{n}
dose	2	6476.57	228 *	25.69	189.07 *
spp	7	540.33	19.02	39.5611	291.83 *
spp x a_w	14	29.56	1.04 ^{n}	0.8556	6.3 *
spp x pH	7	33.95	1.2 ^{n}	1.7627	10.09 *
spp x pres	7	16.29	0.57 ^{n}	0.1993	1.47 ^{n}
spp x dose	14	54.27	1.91 **	2.4616	18.12 *
a_w x pH	2	38.66	1.36 ^{n}	1.5852	11.67 *
a_w x pres	2	13.26	0.47 ^{n}	0.2522	1.86 ^{n}
a_w x dose	4	14.01	0.49 ^{n}	0.9409	6.93 *
pH x pres	1	46.69	1.64 ^{n}	0.0666	0.49 ^{n}
pH x dose	2	2523.4	88.83 *	2.5366	18.67 *
pres x dose	2	25.38	0.89 ^{n}	0.0497	0.37 ^{n}
a_w x pH x spp	14	27.5	0.97 ^{n}	0.6603	4.86 *
pH x pres x spp	7	17.36	0.61 ^{n}	0.0489	0.36 ^{n}
a_w x pH x pres	2	29.3	1.03 ^{n}	0.0392	0.29
a_w x pres x dose	4	29.08	1.02 ^{n}	0.0733	0.54 ^{n}
pH x pres x dose	2	11.86	0.42 ^{n}	0.0414	0.3 ^{n}

* significant at $p < 0.001$
** significant at $p < 0.05$
^{n} no significant $p > 0.05$

I-5 SCREEN OF ANTOXIDANTS FOR CONTROL GROWTH OF SPOILAGE MOULS ON 2% WHEAT FLOUR AGAR

Table I-5 Analysis of variance on the effect of type of antioxidant (anti) dose and species (spp) and their two way interactions on lag times prior to grow and growth rates on bread analogues.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***df***	**MS**	***F***	**MS**	***F***
anti	3	1682.44	215.18 *	2.60279	160.91 *
dose	3	1274.33	162.98 *	2.80588	173.47 *
spp	5	43.06	5.51 *	0.77267	47.77 *
anti x dose	9	338.34	43.27 *	0.36312	22.45 *
anti x spp	15	5.68	0.73 ^{n}	0.02438	1.51 ^{n}
Dose x spp	15	24.13	3.09 **	0.10668	6.59 *

* significant at $p < 0.001$
** significant at $p < 0.05$
^{n} no significant $p > 0.05$

I-6 SCREEN OF ESSENTIAL OILS FOR CONTROL GROWTH OF SPOILAGE MOULS ON 2% WHEAT FLOUR AGAR

Table I-6 Analysis of variance on the effect of type of essential oils (eso) dose and species (spp) and their two way interactions on lag times prior to grow and growth rates on wheat flour agar.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***Df***	**MS**	***F***	**MS**	***F***
eso	19	282.07	47.41 *	0.72047	19.10 *
dose	1	2701.45	454.06 *	1.97381	52.32 *
spp	5	181.26	30.47 *	5.52220	146.39 *
eso x dose	19	282.07	47.41 *	0.72047	19.10 *
eso x spp	95	5.95	1.00 ^{n}	0.03772	1.00 ^{n}
Dose x spp	5	41.86	7.04 *	0.39171	10.38 *

* significant at $p < 0.001$
** significant at $p < 0.05$
^{n} no significant $p > 0.05$

I-7 EFFECT OF DOSE AND TYPE OF ANTIOXIDANT, IN COMBINATION WITH ENVIRONMENTAL CONDITIONS, ON GROWTH OF SPOILAGE MOULDS ON 2% WHEAT FLOUR AGAR

Table I-7a Analysis of variance on the effect of water activity (a_w), pH, type of antioxidants (ant), dose and species (spp) on lag times and colonisation rates.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***df***	**MS**	***F***	**MS**	***F***
spp	5	479.83	28.89 *	5.8427	144.99 *
ant	1	95.97	5.78 **	4.0234	99.84 *
dose	6	10305.61	620.47 *	29.4461	730.73 *
a_w	2	47.2	2.84 ^{n}	3.0727	76.25 *
pH	1	356.85	21.48 *	1.7586	43.64 *
spp x ant	5	74.6	4.49 *	0.2039	5.06 *
spp x dose	30	154.07	9.28 *	0.7031	17.45 *
spp x a_w	10	57.6	3.47 *	0.2626	6.25 *
spp x pH	5	42.25	2.54 **	0.7426	18.43 *
ant x dose	6	67.51	4.06*	0.8963	22.24 *
ant x a_w	2	34.27	2.06 ^{n}	0.3218	7.99 *
ant x pH	1	22.54	1.36 ^{n}	0.0452	1.12 ^{n}
dose x a_w	12	17.78	1.07 ^{n}	0.8102	20.11 *
dose x pH	6	21.86	1.32 ^{n}	0.2106	5.23*
aw x pH	2	61.82	3.72 **	0.1723	4.28**
spp x ant x dose	30	52.09	3.14 *	0.1224	3.04 *
spp x ant x aw	10	57.12	3.44*	0.0667	1.66 ^{n}
spp x ant x pH	5	63.36	3.81 **	0.1142	2.83 **
spp x dose x a_w	60	20.88	1.26 ^{n}	0.1322	3.28 *
spp x dose x pH	30	34.04	2.05 **	0.1109	2.75 *
spp x a_w x pH	10	24.46	1.47 ^{n}	0.0849	2.11 **
ant x dose x a_w	12	27.26	1.64 ^{n}	0.0934	2.32 **
ant x a_w x pH	2	70.58	4.25 **	0.0127	0.31 ^{n}
dose x a_w x pH	12	27.75	1.67 ^{n}	0.0485	1.2 ^{n}

* significant at $p < 0.0101$
** significant at $p < 0.05$
^{n} no significant $p > 0.05$

Table I-7b Analysis of variance on the effect of water activity (a_w), pH, type and dose of antioxidant (ant, dose) on lag times and growth of *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC) and strains M453 and PV3 of *Penicillium verrucosum*.

		***F* value for Lag Times (days)**					
Factor	***Df***	*AO*	*ERE*	*PC*	*PR*	*M453*	*PV3*
ant	1	2.08 ^{n}	10.09 **	15.81 *	19.08 *	0.02 ^{n}	0.04 ^{n}
dose	6	107.94 *	1047.37 *	156.34 *	144.74 *	93.18 *	50.06 *
a_w	2	1.07 ^{n}	6.04 **	0.78 ^{n}	8.35 **	1.57 ^{n}	4.9 **
pH	1	0.09 ^{n}	0.16 ^{n}	21.13 *	19.3 *	0.57 ^{n}	2.93 ^{n}
ant x dose	6	4.34 **	3.16 **	5.51 **	8.23 *	1.34 ^{n}	2.06 ^{n}
ant x a_w	2	1.48 ^{n}	5.34 **	2.14 ^{n}	2.34 ^{n}	8.39 ^{n}	6.97 **
ant x pH	1	1.04 ^{n}	3.46 ^{n}	0.46 ^{n}	1 ^{n}	0.64 ^{n}	8.47 **
dose x a_w	12	0.6 ^{n}	1.13 ^{n}	1.68 ^{n}	2.54 **	0.52 ^{n}	1.62 ^{n}
dose x pH	6	0.74 ^{n}	0.26 ^{n}	5.98 *	4.94 **	0.39 ^{n}	50.38 ^{n}
aw x pH	2	0.49 ^{n}	2.76 ^{n}	1.63 ^{n}	3.13 ^{n}	44.38 ^{n}	2.33 ^{n}
ant x dose x a_w	12	0.91 ^{n}	1.88 ^{n}	1.59 ^{n}	1.71 ^{n}	0.92 ^{n}	1.21 ^{n}
ant x a_w x pH	2	1.47 ^{n}	1.18 ^{n}	0.99 ^{n}	0.68 ^{n}	3.03 ^{n}	3.39 ^{n}
dose x a_w x pH	12	0.24 ^{n}	1.22 ^{n}	1.31 ^{n}	2.12 ^{n}	0.79 ^{n}	1.36 ^{n}
		***F* value Growth Rate (mm day^{-1})**					
Factor	***Df***	AO	ERE	PC	PR	M453	PV3
ant	1	20.35 *	104.38 *	10.23 **	37.8 *	2.29 ^{n}	22.37 *
dose	6	200.03 *	494.8 *	102.05 *	331.95 *	128.24 *	53.67 *
a_w	2	16.02 *	34.88 *	1.26 ^{n}	66.24 *	2.8 ^{n}	26.13 *
pH	1	4.83 **	5.67**	107.47 *	173.58 *	0.16 ^{n}	0 ^{n}
ant x dose	6	9 *	32.04 *	2.64 **	12.3 *	1.5 ^{n}	2.92 **
ant x a_w	2	4.31 **	0.14 ^{n}	7.02 **	8.7 **	0.26 ^{n}	3.67 **
ant x pH	1	2.42 ^{n}	0 ^{n}	6.88 **	1.53 ^{n}	0 ^{n}	1.61 ^{n}
dose x a_w	12	7.20 *	19.45 *	4.22 **	10.09 *	0.98 ^{n}	5.29 *
dose x pH	6	1.21 ^{n}	1.01 ^{n}	8.36 *	30.91 *	0.3 ^{n}	1.67 ^{n}
aw x pH	2	0.22 ^{n}	6.24 **	4.7 **	12 *	5.01 **	1.21 ^{n}
ant x dose x a_w	12	0.75 ^{n}	2.19 ^{n}	2.07 ^{n}	2.54 **	1.09 ^{n}	1.08 ^{n}
ant x a_w x pH	2	0.12 ^{n}	2.09 ^{n}	1.23 ^{n}	1.32 ^{n}	3.27 ^{n}	0.5 ^{n}
dose x a_w x pH	12	0.83 ^{n}	5.42 *	2.25 ^{n}	2.52 **	0.99 ^{n}	1.01 ^{n}

* significant at $p < 0.0101$
** significant at $p < 0.05$
^{n} no significant $p > 0.05$

I-8 EFFECT OF DOSE AND TYPE OF ESSENTIAL OILS, IN COMBINATION WITH ENVIRONMENTAL CONDITIONS, ON GROWTH OF SPOILAGE MOULDS ON 2% WHEAT FLOUR AGAR

Table I-8a Analysis of variance on the effect of water activity (a_w), pH, type of essential oil (eso), dose and species (spp) on lag times and colonisation rates.

		Lag time (days)		Growth Rate (mm day^{-1})	
Factor	***df***	**MS**	***F***	**MS**	***F***
spp	5	594.9	49.45 *	8.587	138.54 *
eso	3	98.6	8.2 *	0.5409	8.73 *
a_w	2	2101.4	174.68 *	22.1713	357.7 *
pH	1	1210.5	100.62 *	4.5189	72.91 *
dose	6	19159.7	1592.62 *	56.039	904.11 *
spp x eso	15	60.5	5.03 *	0.3243	5.23 *
spp x a_w	10	32.0	2.66 **	1.1063	17.85 *
spp x pH	5	58.3	4.85 *	1.4282	23.04 *
spp x dose	30	80.5	6.69 *	0.8493	13.7 *
eso x a_w	6	7.8	0.65 ^{n}	0.127	2.05 ^{n}
eso x pH	3	7.6	0.63 ^{n}	0.0250	0.4 ^{n}
eso x dose	18	12.5	1.04 ^{n}	0.0620	1.0 ^{n}
a_w x pH	2	11.4	0.95 ^{n}	0.2203	3.55 **
a_w x dose	12	264.3	21.97 *	1.9684	31.76 *
pH x dose	6	158.7	13.19*	0.2541	4.1 *
spp x eso x a_w	30	16.1	1.34 ^{n}	0.0801	1.29 ^{n}
spp x eso x pH	15	8.4	0.7 ^{n}	0.0479	0.77 ^{n}
spp x eso x dose	90	16.8	1.4 **	0.0632	1.02 ^{n}
eso x a_w x pH	6	21.8	1.81 ^{n}	0.0504	0.81 ^{n}
eso x a_w x dose	36	8.6	0.72 ^{n}	0.0373	0.6 ^{n}
a_w x pH x dose	12	33.0	2.75 *	0.1961	3.16 *

* significant at $p < 0.0101$
** significant at $p < 0.05$
^{n} no significant $p > 0.05$

Table I-8b Analysis of variance on the effect of water activity (a_w), pH, type and dose of antioxidant on lag times and growth of *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC) and strains M453 and PV3 of *Penicillium verrucosum*.

		***F* value for Lag Times (days)**					
Factor	***Df***	*AO*	*ERE*	*PC*	*PR*	*M453*	*PV3*
eso	3	13.78 *	13.74 *	13.62 *	2.83 **	8.05 *	2.93 **
aw	2	24.23 *	67.34 *	44.65 *	47.77 *	122.14 *	32.21 *
pH	1	37.79 *	9.44**	103.71 *	54.28 *	12.71 *	3.79 ^{n}
dose	6	351.92 *	360.14 *	541.11 *	434.43 *	963.63 *	280.13 *
eso x aw	6	0.93 ^{n}	3.65 **	0.76 ^{n}	0.43 ^{n}	6.15*	1.48 ^{n}
eso x pH	3	2.10 ^{n}	0.55 ^{n}	0.93 ^{n}	0.39 ^{n}	1.24 ^{n}	1.12 ^{n}
eso x dose	18	2.83 **	3.56 *	1.57 ^{n}	1.09 ^{n}	3.57 *	0.9 ^{n}
aw x pH	2	1.05 ^{n}	0.95 ^{n}	2.68 ^{n}	0.75 ^{n}	3.23 **	0.23 ^{n}
aw x dose	12	9.01 *	12.64 *	8.05 *	9.10 *	15.47 *	5 *
pH x dose	6	7.93 *	2.78 **	22.10 *	8.93 *	2.92 **	1.73 ^{n}
eso x aw x pH	6	1.82 ^{n}	1.29 ^{n}	1.77 ^{n}	0.69 ^{n}	2.24 ^{n}	2.86 **
eso x aw x dose	36	0.55 ^{n}	1.86 **	1.23 ^{n}	0.81 ^{n}	2.41*	0.76 ^{n}
aw x pH x dose	12	3.69*	2.14 **	2.34 **	1.69 ^{n}	4.07 *	0.96 ^{n}
		***F* value Growth Rate (mm day-1)**					
Factor	***Df***	AO	ERE	PC	PR	M453	PV3
eso	3	51.29 *	21.78*	9.5 *	2.61 ^{n}	0.58^{n}	5.46 **
aw	2	296.16 *	488.77 *	98.12 *	213.06 *	53.4 *	59.86 *
pH	1	59.65 *	4.43 **	601.22 *	471.38 *	0.29 ^{n}	1.69 ^{n}
dose	6	673.75 *	643.62 *	564.75 *	603.10 *	115.33 *	259.59 *
eso x aw	6	2.84 **	11.62 *	1.72 ^{n}	2.51 **	0.36 ^{n}	3.21 **
eso x pH	3	2.5 ^{n}	3.33 **	1.36^{n}	0.5 ^{n}	0.59 ^{n}	1.22 ^{n}
eso x dose	18	7.07 *	4.7 *	1.31 ^{n}	0.8 ^{n}	0.31 ^{n}	1.02 ^{n}
aw x pH	2	0.05 ^{n}	78.01 *	36.01 *	10.59 *	0.79 ^{n}	0.55 ^{n}
aw x dose	12	27.67 *	55.22 *	11.85 *	27.14 *	6.72 *	4.59 *
pH x dose	6	2.13 ^{n}	9.07 *	54.98 *	57.39 *	0.61 ^{n}	0.56 ^{n}
eso x aw x pH	6	3.04 **	1.63 ^{n}	5 *	1.7 ^{n}	0.46 ^{n}	1.01 ^{n}
eso x aw x dose	36	1.36 ^{n}	2.11 **	1.37 ^{n}	1.48 ^{n}	0.42 ^{n}	0.62 ^{n}
aw x pH x dose	12	2.87 **	24.92 *	9.43 *	2.48 **	1.27 ^{n}	0.51 ^{n}

* significant at $p < 0.0101$
** significant at $p < 0.05$
^{n} no significant at $p > 0.05$

I-9 EFFECT OF ENVIRONMENTAL CONDITIONS ON OCHRATOXIN A PRODUCTION BY DIFFERENT STRAINS OF *PENICILLIUM VERRUCOSUM* ON 2% WHEAT FLOUR AGAR

Table I-9 Analysis of variance on the effect of water activity (a_w), time of incubation (time) and dose of potassium sorbate on ochratoxin A production by strain PV3 of *Penicillium verrucosum* on 2% wheat flour agar.

Ochratoxin A concentration ($\mu g\ g^{-1}$)			
Factor	***df***	**MS**	***F***
a_w	2	198.35	10.79 *
dose	2	381.35	20.74 *
day	5	321.88	17.5 *
a_w x dose	4	134.84	7.33 *
a_w x day	10	78.85	4.29 *
dose x day	10	90.12	4.9 *
a_w x dose x day	20	43.76	2.38 *

* significant at $p < 0.001$

I-10 EFFECT OF ENVIRONMENTAL CONDITIONS ON OCHRATOXIN A PRODUCTION BY DIFFERENT STRAINS OF *PENICILLIUM VERRUCOSUM* ON BREAD ANALOGUES

Table I-10 Analysis of variance on the effect of strain, water activity (a_w), pH and time of incubation (time; days) on ochratoxin A production by *Penicillium verrucosum* on bread analogues with no preservative added.

Ochratoxin A concentration ($\mu g\ g^{-1}$)			
Factor	***df***	**MS**	***F***
strain	2	177280	11.74 *
a_w	2	618699	40.9 *
pH	1	219471	14.54 *
time	4	1159036	76.78 *
strain x a_w	4	85194	5.64 *
strain x pH	2	278037	18.42 *
strain x time	8	214688	14.22 *
a_w x pH	2	565439	37.46 *
a_w x time	8	152369	10.09*
pH x day	4	58244	3.86 **
strain x a_w x pH	4	441693	29.26 *
strain x a_w x time	16	52431	3.47 *
strain x pH x time	8	62330	4.13 *
a_w x pH x time	8	144600	9.58*

* significant at $p < 0.001$
** significant at $p < 0.05$

I-11 EFFECT OF EXISITING PRESERVATIVES, IN COMBINATION WIHT ENVIRONMENTAL CONDITIONS, ON OCHRATOXIN A PRODUCTION BY DIFFERENT STRAINS OF *PENICILLIUM VERRUCOSUM* ON BREAD ANALOGUES

Table I-11a Analysis of variance on the effect of strain, water activity (a_w), pH, strain and type (pres) and dose of existing preservatives on ochratoxin A production by *Penicillium verrucosum* on bread analogues.

Ochratoxin A concentration (μg g^{-1})			
Factor	***Df***	**MS**	***F***
strain	2	433685	34.88 *
pres	1	1781	0.14 ^{n}
a_w	2	312249	25.11 *
pH	1	1319292	106.09 *
Dose	2	925722	74.44 *
Strain x pres	2	8727	0.70 ^{n}
strain x a_w	4	185424	14.91 *
strain x pH	2	209122	16.82 *
strain x dose	4	154992	12.46 *
a_w x pres	2	1467	0.12 ^{n}
Pres x pH	1	4908	0.39 ^{n}
Pres x dose	2	1343	0.11 ^{n}
a_w x pH	2	344416	27.7 *
a_w x dose	4	83647	6.73 *
pH x dose	2	232015	18.66 *
Strain x pres x a_W	4	4064	0.33 ^{n}
Strain x pres x ph	2	5829	0.47 ^{n}
Strain x pres x dose	4	7307	0.59 ^{n}
Strain x a_w x pHh	4	98773	7.94 *
Pres x a_w x pH	2	1537	0.12 ^{n}
Pres x a_w x dose	4	1784	0.14 ^{n}
Pres x pH x dose	2	4001	0.32 ^{n}

* significant at $p < 0.001$
** significant at $p < 0.05$
n no significant at $p > 0.05$

Table I-11b Analysis of variance on the effect of water activity (a_w), pH, strain and type (pres) and dose of existing preservatives on ochratoxin A production by strains M450, M453 and PV3 of *Penicillium verrucosum* on bread analogues.

***F* values -Ochratoxin A concentration (μg g^{-1})**

		STRAIN		
	Df	**M450**	**M453**	**PV3**
pres	1	7.56 **	4.52**	0.82 ^{n}
a_w	2	66.52 *	71.34 *	36.55 *
pH	1	172.45 *	164.46 *	94.49 *
dose	2	345.71 *	80.22 *	70.06 *
a_w x pres	2	0.24 ^{n}	1.51 ^{n}	0.63 ^{n}
pres x dose	1	14.11 *	2.26 ^{n}	0.80 ^{n}
pres x pH	2	1.96 ^{n}	3.05 ^{n}	0.90 ^{n}
a_w x pH	2	4.11 **	87.10 *	23.94 *
a_w x dose	4	11.77*	40.13 *	9.27 *
pH x dose	2	34.27 *	43.98 *	19.7 *
pres x a_w x pH	2	2.5 ^{n}	1.83 ^{n}	0.64 ^{n}
pres x a_w x dose	4	2.62 **	1.57 ^{n}	0.53 ^{n}
pres x pH x dose	2	4.16 **	41.57 *	0.88 ^{n}
aw x pH x dose	4	5.59 **	1.27 ^{n}	11.17 *

* significant at $p < 0.001$
** significant at $p < 0.05$
n no significant at $p > 0.05$

I-12 EFFECT OF ANTIOXIDANTS AND ESSENTIAL OILS ON OCHRATOXIN A PRODUCTION BY DIFFERENT STRAINS OF *PENICILLIUM VERRUCOSUM* ON BREAD ANALOGUES

Table I-12 Analysis of variance on the effect of strain, preservative type (pres ; BHA, PP and essential oils of thyme, clove, cinnamon and bay) and dose on ochratoxin A production by *Penicillium verrucosum* on bread analogues.

Ochratoxin A concentration ($\mu g\ g^{-1}$)			
Factor	***df***	**MS**	***F***
strain	2	6950	5.31 **
pres	5	2313	1.77 ^{n}
dose	1	35929	27.46 *
strain x pres	10	2981	2.28 **
strain x dose	2	23387	17.87 *
pres x dose	5	2313	1.77 ^{n}
strain x pres x dose	10	2981	2.28 **

* significant at $p < 0.001$
** significant at $p < 0.05$
n no significant at $p > 0.05$

I-13 EFFECT OF ENVIRONMENTAL CONDITIONS AND SUB-OPTIMAL CONCENTRATION OF POTASSIUM SORBATE ON NICHE SIZE OF SPOILAGE MOULDS

Table I-13 Analysis of variance on the effect of water activity (a_w) temperature (T), pH and presence of potassium sorbate (pres) on niche sizes of *Aspergillus ochraceus* (AO), *Cladosporium herbarum* (CH), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR) and strains M450, M453 and PV3 of *Penicillium verrucosum*.

		F value							
FACTOR	***df***	AO	CH	ERE	PC	PR	450	453	PV3
pres	1	56.9 **	22.13 **	18.46 **	130.98 *	48.4 **	93.96 *	98.79 **	93.44 **
T	1	90.47 **	1.68 ^{n}	63.18 **	5.44 ^{n}	0.9 ^{n}	0.98 ^{n}	0.42 ^{n}	13.44 **
a_w	3	76.73 **	68.23 **	39.23 **	82.02 **	328.03 *	41.45 **	36.15 **	72.84 **
pH	1	70.86 **	13.92 **	36.18 **	0.11 ^{n}	36.1 **	36.36 **	71.79 **	163.27 *
Pres x temp	1	4.31 ^{n}	3.18 ^{n}	0.42 ^{n}	0.11 ^{n}	14.4 **	7.97 ^{n}	1.7 ^{n}	2.09 ^{n}
pres x a_w	3	5.65 ^{n}	7.45 ^{n}	1.75 ^{n}	4.46 ^{n}	10.47 **	1.82 ^{n}	0.5 ^{n}	0.8 ^{n}
pres x pH	1	90.47 **	15.16 **	81.42 **	89.20 **	25.6 **	82.49 **	122.76 **	125.94 **
T x a_w	3	3.58 ^{n}	1 ^{n}	3.22 ^{n}	2.32 ^{n}	2.97 ^{n}	0.69 ^{n}	2.92 ^{n}	0.28 ^{n}
T x pH	1	8.71 ^{n}	3.18 ^{n}	1.15 ^{n}	2.09 ^{n}	0.1 ^{n}	3.08 ^{n}	2.15 ^{n}	0.31 ^{n}
a_w x pH	3	15.98 **	6.39 ^{n}	2.86 ^{n}	17.13 **	53.5 **	7.94 ^{n}	6.88 ^{n}	2.22 ^{n}
pres x T x a_w	3	2.91 ^{n}	1.13 ^{n}	0.69 ^{n}	0.74 ^{n}	3.13 ^{n}	1.03 ^{n}	1.54 ^{n}	0.64 ^{n}
pres x T x pH	1	10.05 **	1.68 ^{n}	10.38 **	031 ^{n}	3.6 ^{n}	0.7 ^{n}	0.24 ^{n}	0.01 ^{n}
pres x a_w x pH	3	4.92 ^{n}	5.91 ^{n}	4.71 ^{n}	3.17 ^{n}	3.8 ^{n}	0.21 ^{n}	2.18 ^{n}	5.18 ^{n}
T x a_w x pH	3	2.91 ^{n}	1.13 ^{n}	0.45 ^{n}	2.19 ^{n}	6.03 ^{n}	0.21 ^{n}	0.61 ^{n}	0.77 ^{n}

* effect significant at $p < 0.001$
** effect significant at $p < 0.05$
n no significant effect

I-14 EFFECT OF ENVIRONMENTAL CONDITIONS ON TEMPORAL HYDROLYTIC ENZYME PRODUCTION BY SPOILAGE MOULDS ON 2% WHEAT FLOUR AGAR

Table I-14 Analysis of variance on the effect of water activity (a_w), pH, time of incubation and enzyme type (enz) on total and specific activity on cultures of *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR) and strains M453 and PV3 of *Penicillium verrucosum* growing at 25°C on 2% wheat flour agar.

		F value					
		TOTAL ACTIVITY					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
enzyme	2	332.56 *	558.16 *	140.65 *	299.18 *	34.84 *	151.88 *
time	3	39.33 *	57.86 *	52.83 *	136.35 *	42.10 *	104.8 *
a_w	2	87.84 *	30.52 *	1.08 ^{n}	92.88 *	46.74 *	58.3 *
pH	1	10.27 **	87.73 *	135.0 *	39.87 *	23.79 *	2.58 ^{n}
enz x time	6	21.05 *	24.83 *	19.1 *	53.88 *	7.56 *	26.76 *
enz x a_w	4	66.0 *	21.82 *	0.61 ^{n}	10.18 *	15.88 *	10.94 *
enz x pH	2	10.48 *	112.92 *	45.56 *	62.16 *	4.33 **	6.99 *
time x a_w	6	2.46 **	8.67 *	3.04 **	5.49 *	7.47 *	15.64 *
time x pH	3	0.28 ^{n}	5.16 **	19.15 *	15.49 *	4.48 **	19.75*
enz x time x a_w	12	2.82 **	3.9 *	1.5 ^{n}	6.66 *	3.27 *	6.04 *
enz x time x pH	6	0.40 ^{n}	2.88 **	15.0**	9.63 *	2.49 **	7.66 *
		SPECIFIC ACTIVITY					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
enzyme	2	379.33*	559.62 *	167.76 *	300.4 *	52.33 *	121.24 *
time	3	24.49 *	50.64 *	56.66 *	143.37 *	69.94 *	84.11 *
a_w	2	137.8 *	32.4 *	2.35 ^{n}	109.38 *	22.81 *	63.77 *
pH	1	0.01 ^{n}	53.27 *	198.31 *	41.42 *	3.7 ^{n}	0.22 ^{n}
enz x time	6	12.51 *	21.31 *	17.58 *	54.06 *	9.34 *	21.14 *
enz x a_w	4	101.52 *	28.81 *	0.99 ^{n}	10.86 *	10.86 *	11.18 *
enz x pH	2	2.45 ^{n}	81.48 *	59.45 *	57.37 *	0.38 ^{n}	7.01 *
time x a_w	6	6.86 *	11.26 *	3.39 **	5.89 *	5.44 *	11.99 *
time x pH	3	5.53 **	3.84 **	23.24 *	17.86 *	4.16 **	29.43 *
enz x time x a_w	12	5.68 *	4.52 *	1.81 ^{n}	7.36 *	1.84 ^{n}	5.27 *
enz x time x pH	6	2.46 **	2.36 **	8.9**	9.51 *	1.57 ^{n}	9.6 *

* significant effect at $p < 0.001$
** significant effect at $p < 0.05$
n effect not significant at $p > 0.05$

I-15 EFFECT OF PRESERVATIVES AND ENVIRONMENTAL CONDITIONS ON TEMPORAL HYDROLYTIC ENZYME PRODUCTION BY SPOILAGE MOULDS ON 2% WHEAT FLOUR AGAR

Table I-15a Analysis of variance on the effect of water activity (a_w), pH, time of incubation and preservative type (pres) on total and specific activity of α-D-galactosidase by *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR) and strains M453 and PV3 of *Penicillium verrucosum* growing at 25°C on 2% wheat flour agar.

		F value					
		TOTAL ACTIVITY of α-D-galactosidase					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
time	3	115.55 *	6.91 *	33.08 *	99.32 *	47.87 *	12.5 *
a_w	2	152.75 *	14.17 *	14.51 *	55.85 *	5.23 **	12.32 *
PH	1	264.53 *	162.95 *	28.52 *	24.04 *	21.67 *	11.59 *
pres	5	41.05 *	25.93 *	7.57 *	54.24 *	17.25 *	3.65 **
day x a_w	6	16.84 *	6.92 *	9.57 *	23.27 *	25.17 *	14.97 *
day x pH	3	5.43 **	0.32 ^{n}	1.45 ^{n}	2.61 *	12.68 *	17.91 *
day x pres	15	12.77*	2.55 **	5.88 *	20.29 *	10.99 *	3.43 *
a_w x pres	10	10.25 *	7.89 *	10.03 *	21.57 *	20.79 *	4.04 *
pH x pres	5	23.30 *	39.93 *	6.86 *	30.64 *	5.41 *	6.79 *
day x pres x a_w	30	5.00 *	4.48 *	11.44 *	10.12 *	13.91 *	3.97 *
day x pres x pH	15	3.56 *	2.37 **	3.03 *	4.91 *	6.62 *	3.39 *
		SPECIFIC ACTIVITY					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
time	3	43.05 *	5.96 *	32.57 *	98.01 *	41.61 *	10.64 *
a_w	2	103.75 *	13.34 *	10.48 *	75.92 *	5.39 **	13.35 *
pH	1	59.80 *	181.73 *	29.21 *	64.42 *	20.8 *	7.73 **
pres	5	17.27 *	30.57 *	6.6 *	43.99 *	15.13 *	4.17 *
day x a_w	6	28.00 *	6.77 *	7.04 *	31.84 *	23.15 *	10.55 *
day x pH	3	8.01 *	0.71 ^{n}	1.97 ^{n}	3.98 **	9.46 *	13.48 *
day x pres	15	5.19 *	2.39 **	5.06 *	22.23 *	9.88 *	3.38 *
a_w x pres	10	7.32 *	9.63 *	9.97 *	25.41 *	20.66 *	4.6 *
pH x pres	5	7.82 *	52.66*	9.82 *	31.63 *	6.52 *	9.45 *
day x pres x a_w	30	5.08 *	5.49 *	10.16 *	15.47 *	14.81 *	3.63 *
day x pres x pH	15	4.98 *	2.92 *	3.74 *	5.51 *	5.61 *	4.13 *

* significant effect at $p < 0.001$
** significant effect at $p < 0.05$
n effect not significant at $p > 0.05$

Table I-15b Analysis of variance on the effect of water activity (a_w), pH, time of incubation and preservative type (pres) on total and specific activity of β-D-glucosidase by *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR) and strains M453 and PV3 of *Penicillium verrucosum* growing at 25°C on 2% wheat flour agar.

		F value					
		TOTAL ACTIVITY of β-D-glucosidase					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
time	3	68.93 *	12.01 *	356.23 *	179.13 *	40.43 *	23.03 *
a_w	2	19.18 *	8.38 *	3.53 **	23.97 *	288.61 *	62.20 *
pH	1	10.41 *	11.13 *	1973.78 *	11.89 *	359.83 *	35.49 *
pres	5	21.05 *	15.26 *	147.61*	228.66 *	68.77 *	34.93 *
day x a_w	6	3.65 **	4.21 *	1.93 ^{n}	5.25 *	41.06 *	10.84 *
day x pH	3	13.12 *	0.48 ^{n}	238.76 *	20.46 *	40.91 *	6.36 *
day x pres	15	8.42 *	2.43 **	39.82 *	37.49 *	12.31 *	6.63 *
a_w x pres	10	2.22 **	9.99 **	0.62 ^{n}	21.63 *	59.61 *	12.20 *
pH x pres	5	27.01 *	2.49 **	130.79 *	77.85 *	140.60 *	16.15 *
day x pres x a_w	30	2.92 *	3.49 *	0.78 ^{n}	11.08 *	16.72 *	3.24 *
day x pres x pH	15	5.61 *	1.19 ^{n}	46.53 *	24.59 *	36.63 *	9.28 *
		SPECIFIC ACTIVITY					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
time	3	53.57 -	10.38 *	453.92 *	180.41 *	31.77 *	16.82 *
a_w	2	19.72 *	8.36 *	3.93 **	16.09 *	240.16 *	50.65 *
pH	1	25.87 *	15.73 *	2081.13 *	3.05 ^{n}	343.59 *	31.79 *
pres	5	15.86 *	17.24 *	149.73 *	218.52 *	78.88 *	32.67 *
day x a_w	6	6.85 *	4.34 *	2.62 **	3.86 *	28.26 *	7 *
day x pH	3	29.28 *	0.44 ^{n}	315.29 *	22.72 *	34.71 *	6.91 *
day x pres	15	10.92 *	2.58 **	37.74 *	42.22 *	1312 *	5.66 *
a_w x pres	10	3.37 *	10.98 *	0.83 ^{n}	14.71 *	71.02 *	10.86 *
pH x pres	5	28.22 *	3.26 **	119.22 *	76.82 *	196.78*	29.83 *
day x pres x a_w	30	4.10 *	3.88 *	0.81 ^{n}	7.91 *	22.63 *	2.91 *
day x pres x pH	15	7.49 *	1.54 ^{n}	40.98 *	20.59 *	46.48 *	10.01 *

* significant effect at $p < 0.001$
** significant effect at $p < 0.05$
n effect not significant at $p > 0.05$

Table I-15c Analysis of variance on the effect of water activity (a_w), pH, time of incubation and preservative type (pres) on total and specific activity of N-acetyl-β-D-glucosaminidase by *Aspergillus ochraceus* (AO), *Eurotium repens* (ERE), *Penicillium corylophilum* (PC), *Penicillium roquefortii* (PR) and strains M453 and PV3 of *Penicillium verrucosum* growing at 25°C on 2% wheat flour agar.

		F value					
		TOTAL ACTIVITY of N-acetyl-β-D-glucosaminidase					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
time	3	187.69 *	105.79 *	260.03 *	39.01 *	59.98 *	71.7 *
a_w	2	622.07 *	44.29 *	20.36 *	85.39 *	229.22 *	42.33 *
pH	1	374.89 *	32.46 *	846.27 *	612.58 *	210.44 *	0.96 ^{n}
pres	5	68.13 *	411.88 *	114.86 *	60.67 *	64.47 *	83.01 *
day x a_w	6	62.62 *	6.7 *	6.06 *	15.29 *	42.75 *	10.77 *
day x pH	3	63.4 *	11.52 *	87.08 *	85.31 *	36.41 *	3.44 **
day x pres	15	13.33 *	44.56 *	19.07 *	10.24 *	24.49 *	18.33 *
a_w x pres	10	29.26 *	16.23 *	3.6 *	14.61 *	30.8 *	12.84 *
pH x pres	5	56.73 *	85.51 *	66.75 *	122.67 *	66.75 *	22.85 *
day x pres x a_w	30	12.23 *	5.65 *	3.17 *	8.06 *	8.86 *	3.8 *
day x pres x pH	15	13.02 *	8.86 *	28.41 *	21.72 *	23.36 *	7.57 *
		SPECIFIC ACTIVITY					
FACTOR	*df*	AO	ERE	PC	PR	M453	PV3
time	3	145.86 *	90.44 *	375.13 *	35.22 *	39.68 *	55.61 *
a_w	2	598.16 *	48.96 *	24.08 *	65.55 *	219.01*	51.88 *
pH	1	71.12 *	4.25 *	977.08 *	535.18 *	259.20 *	10.18 **
pres	5	55.03 *	356.64 *	159.38 *	70.72 *	76 *	74.43 *
day x a_w	6	43.99 *	8.46 *	9.63 *	14.34 *	35.03 *	11.7 *
day x pH	3	5.54 *	11.45 *	145.49 *	74.28 *	43.27 *	8.59 *
day x pres	15	13.43 *	52.16 *	33.01 *	10.65 *	11.85 *	13.85 *
a_w x pres	10	25.5 *	17.99 *	4.37 *	12.19 *	40.41 *	12.39 *
pH x pres	5	26.73 *	43.12 *	70.91 *	13301 *	118.19 *	21.81 *
day x pres x a_w	30	17.11 *	7.32 *	3.17 *	6.31 *	12.33 *	3.23 *
day x pres x pH	15	11.82 *	15.35 *	37.23 *	21.85 *	34.99 *	7.08 *

* significant effect at $p < 0.001$
** significant effect at $p < 0.05$
n effect not significant at $p > 0.05$

APPENDIX II

LIST OF PUBLICATIONS

1 **Arroyo, M** & Magan, N. (2002). Potential control of spoilage moulds of bakery products using alternative antifungal compounds. *Frontiers in Microbial Fermentation and Preservation*, Joint Meeting of The Society for Applied Microbiology and the Dutch Society for Microbiology, Wageningen, The Netherlands, 9-11 January 2002, p.63-65.

2 **Arroyo, M.**, Cairns, V. & Magan, N. (2001). Impact of preservatives and environmental factors on growth and ochratoxin A production by *Aspergillus ochraceus* and *Penicillium verrucosum* strains on wheat substrates. *British International Symposium on Bioactive Fungal Metabolites: Impact and Exploitation*, Swansea, UK, 22-27 April 2001.

3 **Arroyo, M.** & Magan, N. (2001). Natural antifungal systems for prevention of mould spoilage in bakery products. *Second Postgraduate Conference 2001*, Cranfield University, Silsoe, UK, 21-22 June 2001.

4 **Arroyo, M**. & Magan, N. (2000). Influence of environmental factors and chemical preservatives on growth of common food-borne fungi. *Food Biotechnology: Where Next?*, Institute of Food Science and Technology Symposium, Solihull, UK, 14 April 2000. Best Poster Award.

5 **Arroyo, M.** & Magan, N. (2000). Comparison of interactions between natural and chemical preservatives and environmental factors on activity of spoilage fungi in bakery products. *First Postgraduate Research Conference*, Cranfield University, Silsoe, UK, 28-29 June 2000.

6 Magan, N.; **Arroyo, M.** & Aldred, D. (2003). Natural antifungal agents for bakery products. *In* Natural antimicrobials for minimally processes foods, Edt. S.Roller, Woodhead Editions. ***In Press.***

7 Magan, N.; **Arroyo, M**. & Aldred, D. (2003). Mould prevention in bread. *In* New developments in bread making, Edt. S.Couvain, Woodhead Editions. ***In Press.***

8 Torres, A.M.; Ramirez, M.L.; **Arroyo, M.**; Chulze, S.N. & Magan, N. (2002). Potential use of antioxidants for control of growth and fumonisin production by *Fusarium verticilloides* and *Fusarium proliferatum* on whole maize grain, *International Journal of Food Microbiology*, ***In press.***

(Several Journal papers in preparation)